A Thesis for the Degree of Master of Science

Studies on Application of Spermatogonial Stem Cell for Quail Genome Modification

메추리의 유전자 편집을 위한
정소 줄기세포의 응용 방법에 대한 연구

August, 2019

By
HO YEON CHO

Biomodulation Major
Department of Agricultural Biotechnology
Graduate School, Seoul National University

SUMMARY

Avian species have been considered as one of the most valuable animal models for various applications including developmental biology, disease resistance model and bioreactor. In this circumstance, efforts for generating germline chimeric and transgenic birds have been studied for a long time by a number of investigators. In particular, chicken has been focused as a useful bioreactor model with their high egg production rate. Even, primordial germ cell (PGC), which is one of germline competent stem cells, have been well investigated while long-term *in vitro* culture has been established in chicken. However, it was hard to apply chicken primordial germ cell culture system to other avian species due to lack of cell sources and short in vitro culture duration. Thus, it is necessary to develop alternative germline competent stem cell mediated germline chimeric and transgenic bird generation in other avian species. In this study, we established simple and practical density gradient centrifugation mediated spermatogonial stem cell (SSC) enrichment method, and verified feasibility and enhancement of enriched spermatogonial stem cell's germline transmission efficiency in quail. Furthermore, we induced *in vitro* genome modification in isolated and enriched quail germline competent stem cells with adenoviral vector mediated CRISPR/Cas9 genome editing tool.

From the first study, we enriched quail SSC by density gradient centrifugation methods with Ficoll-Paque PLUS (Ficoll), Percoll and sucrose solutions. To evaluate enrichment of each fractions, expression levels of SSC-specific genes (*GFRA1, ITGA6,* and *ITGB1*) and pluripotency genes (*NANOG* and

POUV) were examined by qRT-PCR. Interestingly, cells from upper fractions in most of density gradients showed significantly higher gene expressions. In addition, qRT-PCR results revealed that cells from upper fractions in Ficoll density gradient showed the highest SSC-specific and pluripotency marker expression. Then we confirmed SSC enriched fractions by RNA hybridization and TEM image. Subsequently, SSC enriched fractions were transplanted into busulfan treated quail testis, and PKH-26 labeled donor cells were detected from the testicular tubules. We performed testcross analysis for verifying germline transmission efficiency. As a results, SSC enriched fraction transplanted quail produced donor-derived sperm and progeny, and its efficiency (8.4 ± 1.7 %) showed significantly 6 times higher than that of whole testicular cells transplanted group (1.4 ± 1.4 %).

Finally, we introduced CRISPR/Cas9 system into quail germline competent stem cells for inducing *in vitro* genomic DNA modification. We transduced embryonic gonadal cells and enriched SSC by adenoviral and adeno-associated viral vectors, and only adenoviral vector showed positive signals in all quail cells. Then we optimized adenoviral transduction with poly-L-lysine adding, and 1 μg/mL concentration of poly-L-lysine mostly optimized adenoviral transduction in all quail cells. For characterizing adenoviral vector transduced cells, we identified germ cell specific (*VASA*, *DAZL*), pluripotency (*NANOG*, *POUV*) and germline stem cell specific (*GFRA1*, *ITGA6*, *ITGB1*) markers from quail PGC, SSC and QM7 cell line. Finally, we constructed adenoviral vector delivering CRISPR/Cas9 targeting for *Transferrin* and *Hoxb13* gene, and induced genome modification in quail germline competent stem cells. T7E1 assay and sequencing

analysis revealed that CRISPR/Cas9 delivery with adenoviral vector induced about 33.3 % of mutation at genomic DNA of quail germline competent stem cells. However, there was no positive signal with adenoviral and adeno-associated viral vectors in chicken germline competent stem cells.

Collectively, these results suggested that Ficoll density gradient solution can be used as a simple and practical method for SSC enrichment, and this method could be applied for bird conservation, restoration and even transgenic quail researches. In addition, adenoviral vector mediated CRIPSR/Cas9 delivering is efficient in quail germline competent stem cells, but not in chicken germline competent stem cells. Thus, we can expect to apply adenoviral vector with CRISPR/Cas9 system via in vitro even in vivo approaches for generating targeted genome edited quail.

Keywords : quail, Ficoll, density gradient centrifugation, spermatogonial stem cell, CRISPR/Cas9, adenoviral vector

Student Number : 2017-26445

CONTENTS

LIST OF FIGURES

CHAPTER 3

CHAPTER 4

LIST OF TABLES

CHAPTER 3

CHAPTER 4

LIST OF ABBREVIATIONS

SSC	Spermatogonial stem cell
PGC	Primordial germ cell
ESC	Embryonic stem cell
HH	Hamburger and Hamilton
EGK	Eyal-Giladi and Kochav
A_s	A single
A_{pr}	A pair
A_{al}	A align
CD	Central disk
GCR	Germinal crescent region
GDNF	Glial cell line-derived neurotrophic factor
GFRA1	*GDNF* receptor alpha 1
ITGA6	Integrin alpha 6
ITGB1	Integrin beta 1
VASA	DEAD-box helicase 4
DAZL	Deleted in azoospermia like
NANOG	Nanog homeobox
POUV	POU domain class 5 transcription factor 3
ACTB	Actin beta
WSP	Wild plumage quail specific primer
BSP	Black plumage quail specific primer
EPCAM	Epithelial cell adhesion molecule
MCAM	Melanoma cell adhesion molecule
SSEA-1	Stage specific embryonic antigen-1
Ig	Immunoglobulin
mAb	Monoclonal antibody

PE	Phycoerythrin
Adv	Adenovirus
CAR	Coxsackie and adenovirus receptor
AAV	Adeno-associated virus
ALV	Avian leucosis virus
SNV	spleen necrosis virus
pLL	Poly-L-lysine
pB	Poly-brene
pEI	Poly-ethyleneimine
FACS	Fluorescence-activated cell sorting
MACS	Magnetic-activated cell sorting
ZFN	Zinc finger nuclease
TALEN	Transcription activator-like effector nuclease
CRISPR/Cas9	Clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR-associate protein 9
crRNA	CRISPR RNA
tracrRNA	Trans-acting CRISPR RNA
PAM	Protospacer adjacent motif
xCas9	Expanded PAM spCas9
dCas9	Cleavage domain mutated Cas9
CMV	Cytomegalovirus
Ficoll	Ficoll-paque PLUS
PCR	Polymerase chain reaction
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
TEM	Transmission electron microscope
ICC	Immunocytochemistry
PBS	Phosphate buffered saline
HBSS	Hank’s balanced salt solution
FBS	Fetal bovine serum

BSA	Bovine serum albumin
DMEM	Dulbecco’s minimum essential medium
DEPC	Diethyl pyrocarbonate

CHAPTER 1

GENERAL INTRODUCTION

Avian species have been regarded as important models for various purposes. Especially, due to their oviparous characteristic it is easy to observe and manipulate avian embryos (Selleck, 1996). According to this characteristic, avian species were regarded as a great model for developmental studies. Even their high egg production rate made them as a valuable model for egg bioreactor as functional protein producing (Herron, et al., 2018). Although chicken has great advantages as a disease resistance and bioreactor model, quail also has its advantage as a developmental studying models because of their relatively short generation period (Ivarie, 2003). However, it is difficult to generate models birds for various purposes in avian species due to lack of practical germline competent stem cell applications, except chicken.

Primordial germ cell (PGC) is the most well investigated germline competent stem cells in avian species. PGC is located on the central zone of area pellucida at Eyal-Giladi and Kochav (EGK) stage X with scattered pattern in avian species (Ginsburg and Eyalgiladi, 1987). As egg is incubated, PGC migrates to germinal crescent at EGK stage 4, and finally settle in genital ridge at EGK stage 12 (Ginsburg and Eyalgiladi, 1989). And this distinct characteristic made it easier to isolate and manipulate avian PGC from diverse embryonic tissues with different stages. There were already many methods developed for isolating avian PGC by surface specific antibody-based fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS). Especially, *in vitro* methods for culture of isolated PGC and even PGC line were established in chicken (Choi, et al., 2010; Macdonald, et al., 2010). There were also several reports about *in vitro* culture of

PGC in quail and duck, but it was hard to maintain as long-term cultivation (Park, et al., 2008; Chen, et al., 2019). Due to lack of sources for PGC and difficulties for handling, there should be alternative germline competent stem cell for bird conservation and transgenic researches in other avian species.

Spermatogonial stem cell (SSC), which has self-renewal and continuous differentiating abilities, is one of germline competent stem cells in adult testis tissue. SSC is normally located at the basement of seminiferous tubules and has a role as a foundation for spermatogenesis in male (Phillips, et al., 2010). SSC is originated from gonocytes in the postnatal testis, which is specified from PGC during embryonic development. After specification, they made balance between self-renewal and differentiating, and this balance maintains the stem cell population and demands for producing a number of sperm in testis. SSC was used for studying spermatogenesis and transgenesis in animals. However, similar to adult stem cells in other tissues, SSC contains only 0.03 % of all germ cells in testis (Tegelenbosch and de Rooij, 1993). Thus it was essential to develop effective methods for enriching and purifying SSC from adult testis. There were a lot of effective purification and *in vitro* cultivation methods for SSC in mouse and human (Sun, et al., 2008; Lim, et al., 2013). However, few studies were reported about SSC in vitro culture in avian species including chicken, quail and pheasant, but it was also hard to maintain as long-term cultivation (Kim, et al., 2014; Momeni-Moghaddam, et al., 2014; Pramod, et al., 2017). Thus it is essential to enhance SSC enrichment and purification for practical applications in avian species.

Programmable genome editing technology, which is tool for artificially modifying desired target sequences at genomic DNA of organisms, is considered as promising biotechnology at present (Kim and Kim, 2014). The best advantage of programmable genome editing is precise recognition and efficient cleavage of double strand DNA unlike existing transposable elements. By using this technique, we can induce not only loss-of-function but also gain-of-function by homologous recombination (Li and Heyer, 2008). And many applications with programmable genome editor variants could be applied. There have been three generations in programmable genome editing tools. At first, zinc finger nuclease (ZFN) emerged, then transcription activator-like effector nuclease (TALEN) and finally clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR-associate protein 9 (CRISPR/Cas9) system have been developed in order. The best advantage of CIRPSR/Cas9 system is simplicity of synthesizing guide RNA compared to previous genome editing tools, and because of its simplicity, CRISPR/Cas9 system have been explosively used for various researches. In addition, their following applications are also actively studied even in avian biotechnology.

Adenoviral vectors were initially applied for human gene therapy, because of their non-integrating characteristic and high level of transgene delivery efficiency in human. Adenovirus offer significant advantages for gene therapy compared to other viral gene delivery systems. Unlike retroviral vectors, which can infect only dividing cells, adenoviral vectors have relatively high transduction efficiency in both dividing and non-dividing cells (Takehashi, et al., 2007). In addition, most of human cells express primary adenovirus receptor and secondary

integrin receptors, adenoviral vectors show broad tropism for *in vivo* applying to human. Thus, adenoviral vector can easily infect target cells and yield high level of transgene expression (Crystal, 2014). Furthermore, with combination of CRISPR/Cas9 system, adenoviral vector can deliver programmable genome editing tool efficiently to broad range of primary cells without integration, which may be able to induce off-target effects. Therefore, non-integrating adenoviral vector could be a great vehicle for delivering CRISPR/Cas9 into quail germline competent stem cells i*n vitro*.

In this study, we demonstrated applications of quail SSC with density gradient centrifugation, and CRISPR/Cas9 delivery with adenoviral vectors *in vitro* as a basic investigation for targeted genome modified quail production. In CHAPTER 2, we reviewed germline competent stem cells and their applications with CRISPR/Cas9 system in animals including mammalian and avian species. In CHAPTER 3, we demonstrated Ficoll density gradient centrifugation mediated SSC enrichment and its practical applications for germline chimeric quail production. In CHAPTER 4, we demonstrated CRISPR/Cas9 delivery to primary quail and chicken germline competent stem cells with adenoviral vectors *in vitro*. Our study has worthy as revealed feasibility and possibility of practical applications for transplantation methods as bird conservation model and a first report of adenoviral vector mediated *in vitro* genomic DNA modification in quail germline competent stem cells by CRISPR/Cas9.

CHAPTER 2

LITERATURE REVIEW

1. Quail as a research model bird

Avian species have been studied as valuable models in biotechnology for various purposes, including developmental, bioreactor, disease resistance and human disease models. Because of their oviparous characteristic, avian species are suitable model for developmental studies, as their embryos could be easily manipulated *in ovo* (Selleck, 1996). Furthermore, avian models were considered as a suitable model for egg bioreactor system by using their egg protein secretion via oviduct (Park, et al., 2014; Oishi, et al., 2016). Actually these kinds of studies were specifically accomplished in chicken, but quail also has a number of merits as an animal model. Because of its relatively short generation period, reproducing rate, high egg production index and small body size, quail could be used as an appropriate developmental and inherited disease models (Ivarie, 2003). However, for accomplishing these kinds of researches, there should be developed more efficient and practical germline chimeric or transgenic quail production methods at present.

1.1. Quail as a bird conservation model

Germline chimeric quails were regarded as suitable model for bird conservation and restoration studying models. And there were several studies about germline chimeric quail production via transplantation of exogenous germline competent stem cells. At first, quail primordial germ cell (PGC) from embryonic gonads were purified by specific surface marker, and they were transplanted into embryonic blood vessel at Hamburger and Hamilton (HH) stage 14 to 16. And they successfully produced germline chimeric quail with 1.9 and 2.2 to 4.7 % of germline transmission efficiencies (Kim, et al., 2005). Even with 20 days of *in vitro* cultivation,

quail gonadal PGC was transplanted, and successfully produced donor-derived progeny with efficiencies of 33.3 and 50 % (Park, et al., 2008). Although PGC-mediated germline chimeric quail production methods were successful, they have limitations for applying other endangered bird species because of few amounts of donor cell sources and difficulties in in vitro culture system establishment. Thus, spermatogonial stem cell (SSC) was emerged as one of alternative cells sources for germline chimeric quail production. Germline chimeric quail was produced by *in vitro* cultured testicular cells transplantation, and resulted 0 and 16.7 % of germline transmission efficiencies (Kim, 2018). Even there were efforts for interspecific transplantation of germline competent stem cells between quail and chicken. Embryonic stem (ESC) central disk (CD) of the area pellucida in quail blastoderm at stage X and germinal crescent region (GCR) of quail embryo at stage 7 to 8 were transplanted into sub-germinal cavity of chicken blastoderm at stage X, and quail genome was detected from 6/55, and 8/68 hatched chicken embryos respectively (Soh, et al., 2004). Moreover, xenogeneic transplantation of quail embryo and SSC into chicken embryo or embryonic blood vessel at stage 7 to 8 resulted quail cell migration at chicken embryonic gonads, respectively (Roe, et al., 2013; Choi, et al., 2015).

1.2. Quail as a developmental biology model

There were several studies about production of transgenic quails by lentiviral vector. At first, tissue-specific transgenic quail was produced by simple lentiviral vector injection into sub-germinal cavity driven by human synapsin gene I promoter. Transgenic quails showed GFP signal only at axons and dendrites of

neurons in vivo, and this kind of model could provide powerful tool for developmental and behavioral neurobiology (Scott and Lois, 2005). And then, researchers generated transgenic quail with lentiviral vector injection into various germline competent cell sources including blastoderm, blood vessel and even in cultured quail PGC (Shin, et al., 2008; Poynter, et al., 2009; Zhang, et al., 2012). The transgenic quail production efficiencies were lower at blastodermal injection (1.7, 1.9 %) and cultured PGC (1.6 %), but it has quite enhanced at blood vessel injection experiments as showing 13 % of efficiency. Although these kinds of efforts, it is still limited for producing transgenic quail with diverse desired models in developmental biology. There were only simple GFP visualizing models of nervous system and embryogenesis in developed transgenic quail (Seidl, et al., 2013; Huss, et al., 2015).

2. Germline competent stem cell in avian biotechnology

Germline competent stem cell is unique cell source which has ability of transferring genetic information to next generation. It contains not only endogenous germline stem cells including embryonic stem cells (ESC), primordial germ cell (PGC) and SSC, but also in vitro cultured germline stem cells (Han, et al., 2015). Modification and engineering genomic DNA of germline competent stem cells have been used as strategies for production of genome-edited organisms. In avian species, several types of germline competent stem cells have been used for applications in biotechnology. It has been suggested that blastodermal cells at stage X show similar characteristics with mammalian ESCs as maintaining pluripotency and undifferentiated status from blastocysts (Eyal-Giladi and Kochav, 1976). And many researchers tried to increase germline transmission efficiency of blastodermal cells

transplantation methods, but they it showed relatively low efficiencies (Petitte, et al., 1990; Carsience, et al., 1993; Pain, et al., 1996). To overcome this limitation, PGC was actively studied as an alternative cell source for avian biotechnology (Tajima, et al., 1993; Naito, et al., 1994; Han, et al., 2002). PGC studies in avian species were focused on chicken, and only chicken PGC could be long-term cultured in vitro (Choi, et al., 2010; Macdonald, et al., 2010; Naito, et al., 2015). However, there were few studies about quail PGC, and it showed also low germline transmission efficiency (Ono, et al., 1998).

2.1. Primordial germ cell (PGC)

In avian species, PGC is located in the area pellucida, the central region of Eyal-Giladi and Kochav (EGK) stage X embryos with scattered pattern (Eyal-Giladi and Kochav, 1976). And PGC migrates to germinal crescent between Hamburger and Hamilton (HH) stage 2 and 4 (Hamburger and Hamilton, 1951). Then PGC moves into blood vessel, and finally settles in the genital ridge through blood stream between HH stage 9 and 12 (Meyer, 1964). So avian PGC can be obtained from specific stages and locations in embryo, and it makes easier PGC obtaining and manipulation in avian system. Avian PGC has been isolated from embryonic tissue or blood vessel by density gradient centrifugation and antibody-based methods including fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS) in quail and chicken (Chang, et al., 1992; Ono and Machida, 1999; Mozdziak, et al., 2005). PGC specific surface antibodies were reported while PGC was also considered as a suitable cell source for bird conservation studies, and there were several studies reported about interspecific

germline chimeric birds (Reynaud, 1969; Kang, et al., 2008; Wernery, et al., 2010; Liu, et al., 2012; van de Lavoir, et al., 2012).

PGC-mediated transgenesis was firstly tried by avian leucosis virus (ALV) (Salter, et al., 1986). However, non-purified PGC mediated transgenesis showed low germline transmission efficiency. At present, long-term in vitro culture of chicken PGC was established (Choi, et al., 2010; Whyte, et al., 2015), and it has become more efficient to produce transgenic chickens with various purposes by introducing transposable elements and programmable genome editing platforms with drug selection (Macdonald, et al., 2012; Park and Han, 2012; Dimitrov, et al., 2016; Lee, et al., 2016; Oishi, et al., 2016). There was also only one report about production of *in vitro* cultured gonadal PGC mediated germline chimeric quails. Moreover, in vitro cultured quail PGC maintained germline competency more than 20 days (Park, et al., 2008). However, PGC mediated germline chimeric birds have limitations as essential *in vitro* culture system, and low germline transmission efficiencies of interspecific applying. It is due to small number of donor cells and developmental differences between bird species, respectively.

2.2. Spermatogonial stem cell (SSC)

Spermatogonial stem cell (SSC), which has self-renewal and continuous differentiating abilities, is one of germline competent stem cells in adult testis tissue. It is normally located at the basement of seminiferous tubules and has a role as a foundation for spermatogenesis in male (Phillips, et al., 2010). SSC is originated from gonocytes in the postnatal testis, which is specified from PGC during

embryonic development. After specification, they made balance between self-renewal and differentiating, and this balance maintains the stem cell population and demands for producing a number of sperm in testis. In spermatogenesis process, A single (A_s) cell, which represents SSC, could be proliferate as independent A_s cell or divided into A pair (A_{pr}) cells with intercellular bridges (Oakberg, 1971). With subsequent spermatogenesis, A aligned (A_{al}) cells were divided as A-type spermatogonia (A1 to A16) and differentiated into B-type spermatogonia after 6 to 7 additional divisions. And finally they were matured as a sperm passing through primary and secondary spermatocyte state with specific micro-environmental conditions. Especially, sertoli cells were essential for controlling SSC self-renewal and continuous spermatogenesis (Jegou, 1993).

SSC was considered as an important source for applications of animal transgenesis and clinical aspects (Goossens, et al., 2013). SSC was used for studying spermatogenesis and transgenesis in animals. However, similar to adult stem cells in other tissues, SSC contains only 0.03 % of all germ cells in testis (Tegelenbosch and de Rooij, 1993). Thus it was essential to develop effective methods for enriching and purifying SSC from adult testis. So there were several studies about characterizing and isolating SSC from adult testis.

Morphologically, SSC in mouse testis were characterized as their round shape, clear cytoplasm and diameter of 14 to 16 μm. They showed relatively larger size as easily distinguishable from sertoli cells with diameter of 8 μm (Kokkinaki, et al., 2009). And SSC has normally relative low density compared to other

differentiated and somatic cells. Because, relative size of SSC is reduced, while maintaining cellular mass through differentiation procedure (Goodyear and Brinster, 2017). However, because of their rareness and continuously differentiating characteristic, it is hard to precisely identify SSC by superficial way (Aponte, et al., 2005). However, using high resolution microscopy and specifically fixed methods, it is more easier to observe subtle differences between SSC and differentiated cells (Chiarini-Garcia and Russell, 2001). In contrast to more differentiated type A spermatogonia, SSC contain low electron dense of heterochromatin at the rim of nucleus (Maezawa, et al., 2018). In case of fish, like mammals, there were no heterochromatin at SSC and it reached maximum density from late stage type B spermatogonial cells (Lacerda, et al., 2014). Nevertheless, even with morphological differences and well trained-eye, identification of SSC only by morphological characteristic is extremely difficult. Thus, there were several studies about molecular characteristics on SSC with purpose for purification and identification of SSC.

ITAG6 and *ITGB1*, one of the laminin receptors, were firstly suggested as specific surface molecules on SSC. And *ITGA6*- and *ITGB1*-mediated selection demonstrated 8.4 and 3.8-fold enrichments of SSC (Shinohara, et al., 1999). And *GFRA1* (*GDNF* receptor alpha 1) was also suggested as a SSC specific surface molecule, because knockout studies revealed that glial cell line-derived neurotrophic factor (*GDNF*) and its receptor *GFRA1* are essential for SSC self-renewal (Meng, et al., 2000). Furthermore, the embryonic stem cell marker (*CD9*), epithelial cell adhesion molecule (*EPCAM*), melanoma cell adhesion molecule (*MCAM*) and their combinations were suggested as SSC specific surface molecules and they exhibited

maximum 560.5-fold higher enrichment of functional SSC (Kanatsu-Shinohara, et al., 2004; Ryu, et al., 2004; Kanatsu-Shinohara, et al., 2012). In addition, SSC was isolated by FACS-based method, from *OCT4*-GFP conjugated transgenic mice (Ohmura, et al., 2004).

2.3. Transplantation of SSC in animals

Transplantation of SSC was designed at 1994 and actively studied in mammals (Brinster and Avarbock, 1994). After germ cell transplantation system was established, studies of spermatogenesis and transgenesis in animal were promoted. And this approach was also confirmed as transplanted donor cells generating functional gametes in mouse and rat testis (Brinster and Zimmermann, 1994; Ogawa, et al., 1999). Then researchers applied SSC transplantations into various species including cattle, goat, dog and chicken (Izadyar, et al., 2002; Honaramooz, et al., 2003; Herrid, et al., 2006; Lee, et al., 2006a; Kim, et al., 2008). These kinds of SSC transplantation studies opened up possibilities of transgenesis in various animal species.

To enhance donor-derived progeny production efficiencies, endogenous germ cell depletion was suggested. Irradiation with gamma-rays or X-rays was suggested as an effective method for depletion of endogenous cells in testis. Although irradiation has risk of non-specific lethality to all types of testicular cells, it worked effectively to spermatogonial transplantation as reducing endogenous spermatogenic cells in various species including mouse, cattle, sheep and chicken (Izadyar, et al., 2003; Trefil, et al., 2006; Herrid, et al., 2009; Koruji, et al., 2012).

Busulfan, which is anti-spermatogonial alkylating reagent, was also introduced for depletion of endogenous germ cells. Busulfan induced spermatogonial cell depletion in testis, resulting reduced sized testes in japanese quail (Jones, et al., 1972). Because of its specificity, lethality of non-spermatogonial cells were reduced and widely used for enhancing SSC transplantation efficiency in other animals including rat, pig and chicken (Boujrad, et al., 1995; Tagirov and Golovan, 2012; Lin, et al., 2017).

According to SSC transplantation methods were enhanced, transgenic animal production methods via SSC modification were also developed. Lentivirus was widely used because of its broad tropism and integrating ability. Lentiviral vector transduced rat SSC was transplanted into heterologous testes, and successfully produced transgenic progeny (Hamra, et al., 2002). And this strategy was also applied for production of transgenic tree shrew (Li, et al., 2017). Furthermore, more elaborate genome modification with transposon was introduced into SSC transplantation methods, and knockout rats were generated successfully (Izsvak, et al., 2010). In recent, TALEN and CRISPR/Cas9 systems were introduced into mouse SSC to induce spermatogenic failure for studying spermatogenesis (Sato, et al., 2015). Even, CRISPR/Cas9 mediated knockout mouse was generated by SSC transplantation (Wang, et al., 2017). Thus, by using the combination of SSC transplantation and various genome editing tool it may possible to produce transgenic animals even in avian species.

2.4. Applications of SSC in avian species

There were many reported surface and specific markers for SSC in mammals, but not well studied in avian species. So it is needed to alternative SSC isolating methods for applications in avian biotechnology. Several methods were reported for enriching, purifying and even *in vitro* culturing SSC in various species including mouse, pig, cattle and human (Nagano, et al., 2003; Kossack, et al., 2009; Kuijk, et al., 2009; Nasiri, et al., 2012). And by using SSC's distinct characteristic, differential plating (Giassetti, et al., 2016), density gradient centrifugation (Liu, et al., 2011; Panda, et al., 2011), and antibody-mediated purification methods, such as fluorescence activated cell sorting (FACS) (Shinohara, et al., 2000) and magnetic cell sorting (MACS) were suggested as a representative SSC isolating methods (Buageaw, et al., 2005). The differential plating and even *in vitro* cultivation of quail and chicken SSC were reported previously (Jung, et al., 2007; Momeni-Moghaddam, et al., 2014; Pramod, et al., 2017). In vitro cultured avian SSC showed SSC-specific, germness-related, stemness-related markers, but maintained with short period. Then, germline chimeric quail was produced by transplantation of *in vitro* cultured quail SSC, and resulted 0 and 16.7 % of germline transmission efficiencies (Kim, et al., 2018). However, it is hard to apply previous studies about avian SSC for transgenic researches and even bird conservation studies, because of short term in vitro duration and varied germline transmission efficiencies. Therefore, it is needed to more enhance current avian SSC mediated germline chimeric and transgenic bird production systems.

3. Programmable genome editing technology

Programmable genome editing technology, which is tool for artificially modifying desired target sequences at genomic DNA of organisms, is considered as promising biotechnology at present (Kim and Kim, 2014). The best advantage of programmable genome editing is precise recognition and efficient cleavage of double strand DNA unlike existing transposable elements. By using this technique, we can induce not only loss-of-function but also gain-of-function by homologous recombination (Li and Heyer, 2008). And many applications with programmable genome editor variants could be applied.

3.1. Zinc finger nuclease (ZFN)

Zinc finger nuclease (ZFN) is first generation programmable genome editing tool. ZFN is composed of two elements, zinc finger motif and FokI nuclease. Zinc finger motif binds to specific DNA sequence, and FokI endonuclease work as breaking double stranded DNA (Kim, et al., 1996). According to amino acid composition, zinc finger motif can bind to specific three nucleotides, and it is more effective to use pair of ZFN for effective DNA cleavage (Smith, et al., 2000). And it is normally used as combination of 6 to 8 ZFN for detecting specific 20 nucleotides. Furthermore it has been widely used for genome modification not only in animal cells but also in plant cells (Urnov, et al., 2010). However, it was revealed that ZFN has unexpected cleavage effects *in vitro* and *in vivo* (Gabriel, et al., 2011; Pattanayak, et al., 2011).

3.2. Transcription activator-like effector nuclease (TALEN)

Transcription activator-like effector nuclease (TALEN) is regarded as second generation programmable genome editing tool. Similar to ZFN, TALEN also composed of two elements containing TAL effector and FokI nuclease. TAL effector is proteins secreted from *Xanthomonas* bacteria for infecting various plant species (Boch, et al., 2009). And TAL effector can act like zinc finger motifs, recognizing and binding to specific DNA nucleotides. DNA binding domain from TAL effector can be consisted of 33 to 35 conserved amino acids residue and Repeat Variable Di-residue. The binding affinity of TAL effectors to specific nucleotide sequences are determined by amino acid combinations in RVD (Li, et al., 2013). Unlike ZFN, TALEN needs only one repeat domain for binding specific nucleotides. This makes TALEN as a better tool for DNA cleavage, since diverse target sites could be designed (Miller, et al., 2011). TALEN also has been used for various organisms (Mussolino and Cathomen, 2012), but it was laborious for synthesizing compared to next generation programmable genome editing tool.

3.3. Clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR-associate protein 9 (CRISPR/Cas9)

Clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR-associate protein 9 (CRISPR/Cas9) system is third generation programmable genome editing tool. CRISPR/Cas9 system is originated from bacterial immune system, which induces disruption of invaded viral genome with Cas9 protein. Similar to eukaryotic animals' immune system, they possess identical sequence with viral genome at usual time, and when viral genome is invading, RNA

and Cas9 protein complex disrupts viral integration site (Barrangou, et al., 2007). Basic principle of CRISPR/Cas9's target DNA cleavage is almost same as ZFN and TALEN. For recognizing specific nucleotide sequences, CRISPR RNA (crRNA) and trans-acting RNA (traRNA) helps Cas9 protein, and Cas9 protein cleave double stranded DNA (Jinek, et al., 2012). The best advantage of CIRPSR/Cas9 system is simplicity of synthesizing guide RNA, but protospacer adjacent motif (PAM) sequence should be present beside target sites for Cas9 activity (Mojica, et al., 2009). In recent there are many variants of Cas9 protein for various purposes. It contains smaller Cas9 protein such as saCas9 and cjCas9, Cpf1 lacking tracrRNA, and even expanded PAM spCas9 (xCas9) can recognize broad range of PAM sequences including NG, GAA and GAT (Ran, et al., 2015; Hur, et al., 2016; Kim, et al., 2017a; Hu, et al., 2018). Moreover cleavage domain mutated Cas9 (dCas9) has been used for various purposes with transcription activators or repressors, and DNA nickase or base-editors (Gaudelli, et al., 2017; Liao, et al., 2017; Satomura, et al., 2017; Fernandes, et al., 2019).

3.4. Programmable genome editing in avian species

As programmable genome editing technology rapidly developed, there have been reported a lot of studies about applying editing tool for animal models. Even for avian species, CRISPR/Cas9 mediated targeted genome editing was accomplished in chicken and quail somatic cell line (Veron, et al., 2015; Abu-Bonsrah, et al., 2016; Ahn, et al., 2017). However, for more practical applications with transgenic avian model, in vitro culture of germline competent stem cells or efficient germline chimeric animal production methods were necessary. In case of

chicken, PGC culture systems were well established and there were several reports about transgenic chicken with CRISPR/Cas9 system (Dimitrov, et al., 2016; Oishi, et al., 2016). There were only reports about efficient *in vitro* germline competent stem cell culture in chicken. In case of quail, CRISPR/Cas9 system was delivered by adenoviral vector via blastodermal injection, and induced production of genome modified quail (Lee, et al., 2019). However, there were no reports about production of targeted genome modified birds in other avian species. So, as following development of genome editing tools, germline competent stem cell studies in other bird species should be accomplished.

4. Adenoviral vector

Adenovirus (Adv) is DNA virus with approximately 3.6 kb of viral genome encompassed by icosahedral protein capsid. And its life cycle is composed of largely two parts as early phase, which occurs initiation of viral DNA prior to replication, and late phase, which occurs following the initiation of DNA replication. The early phase proteins are mainly regulatory proteins for viral DNA replication, and late phase proteins are structural proteins of virus (Douglas, 2007). Adenovirus, named after human adenoids where cytopathogenic agent was isolated from at first (Rowe, et al., 1953), and more than 50 serotypes were reported in adenovirus (Wilson, 1996). Adenovirus has emerged as effective gene delivery vector, because of well-defined biological information, genetic stability, high gene transduction efficiency and ease of large-scale production (Crystal, 2014).

4.1. Adenoviral vector as a tool for gene therapy

Adenoviral vectors were initially applied for human gene therapy, because of their non-integrating characteristic and high level of transgene delivery efficiency in human. Adenovirus offer significant advantages for gene therapy compared to other viral gene delivery systems. Unlike retroviral vectors, which can infect only dividing cells, adenoviral vectors have relatively high transduction efficiency in both dividing and non-dividing cells (Takehashi, et al., 2007). In addition, most of human cells express primary adenovirus receptor and secondary integrin receptors, adenoviral vectors show broad tropism for *in vivo* applying to human. Thus, adenoviral vector can easily infect target cells and yield high level of transgene expression (Crystal, 2014). However, adenovirus has its limitation for applying to gene therapy in human as their immunogenicity. All adenoviral proteins showed immune response in human, and this immunity largely hinders the efficiency gene delivery (Vannucci, et al., 2013). Thus, investigators have found rare serotype 2 and 5 of adenovirus, which is not prevalent in general population (Lasaro and Ertl, 2009).

There were several improvements in use of adenoviral vectors which are mostly derived from human adenovirus serotype 5. First generation adenoviral vectors were made by elimination of viral replication gene. E1a and E1b which are regulatory factors for viral replication during infection cycle were eliminated for increasing transgene cloning capacity as 5.2 kb (Rauschhuber, et al., 2012). In addition, second generation adenoviral vectors were made with additional elimination of non-structural genes (E2, E3, E4). According to this modification,

adenoviral vector demonstrated increased cloning capacity and reduced cytotoxicity (Rauschhuber, et al., 2012). Third generation adenoviral vectors are called as high-capacity adenoviral vectors or helper-dependent adenoviral vectors. All viral coding sequences were eliminated except 5' and 3' ITRs with packaging signal sequences in third generation adenoviral vectors. This elimination provides larger capacity of cloning transgenes and reduce immunogenicity with maximum efficiency. However, for producing third generation adenoviral vectors, trans-acting complementary or helper viruses are essential (Parks, et al., 1996). At present, adenoviral vectors mediated gene therapy was widely used for monogenic disease, vaccine, anti-cancer agents and even DNA regenerative medicine (Lee, et al., 2017).

4.2. Adenoviral vector mediated transgenesis in animals

Few studies were reported about adenoviral vector mediated transgenesis into germline competent cells, because there were already powerful gene delivery tools such as lentiviral vector, electroporation and liposomes for *in vivo* and *in vitro*. Moreover, adenovirus's non-integrating characteristic was disadvantage for tracing transgenes like GFP. Furthermore, there were reports about no sign of infection from spermatogenic cells and mature sperm after direct in vivo injection of adenoviral vector into testis, but only sertoli cells preferentially infected (Hall, et al., 2000; Peters, et al., 2001). Nevertheless, adenoviral vectors were applied into male germline stem cells or mature sperm for the purpose of spermatogenic disease and male infertility. Testicular cells from adenovirus sensitive strain were incubated *in vitro*, and high titration of Cre-expressing adenoviral vector induced generation of transgenic mice with Cre-mediated LacZ gene insertion (Kanatsu-Shinohara, et al.,

2002). In addition, for transgenesis studies, adeno-associated viral vectors were widely used in gene therapeutic aspect. Because, they have tissue specificity and low immunogenic responses. Thus, there were several studies about adeno-associated viral vector mediated germline transgenesis in mouse and pig (Honaramooz, et al., 2008; Zeng, et al., 2013; Watanabe, et al., 2018).

4.3. Adenoviral vector mediated CRISPR/Cas9 delivery in animals

From basic science to clinical applications, adenoviral vector mediated genetic modification opened new areas of investigation. Due to its large capacity, non-integrating character and high efficiency of transgenesis, adenoviral vector matched as a suitable vehicle for delivering programmable genome editing tool *in vivo* and *in vitro*. Even some investigators constructed single vector system for CRISPR/Cas9 delivering adenoviral vectors with enhanced efficiency (Ehrke-Schulz, et al., 2017; Schiwon, et al., 2018; Jin, et al., 2019). There were several efforts for delivering cutting-edge genome editing tool, CRISRP/Cas9 system, into human and mouse cells with adenoviral vectors (Cheng, et al., 2014; Maggio, et al., 2014; Gwiazda, et al., 2016; Voets, et al., 2017). In addition, *in vitro* delivery of CRISPR/Cpf1 was also conducted by adenoviral vectors and they showed high efficiencies of transduction and genome editing in human cells (Tsukamoto, et al., 2018). Recently, adenoviral vector mediated direct delivery of CRISPR/Cas9 into blastoderm in quail egg resulted generation of transgenic quail (Lee, et al., 2019). Thus, the broad tropism and high transgenesis efficiency of adenoviral vectors can be expected as a great vehicle for delivering CRISPR/Cas9 system into avian primary cells or germline cells which are hard to transfect *in vitro* or *in vivo*.

CHAPTER 3

ENRICHMENT OF SPERMATOGONIAL STEM CELL BY DENSITY GRADIENT CENTRIFUGATION AND ITS TESTICULAR TRANPLANTATION FOR GEMRLINE TRANSMISSION

1. Introduction

As one of the important part for life-supporting systems on earth, biodiversity is important because of its direct or indirect effects for well-being of humans (Tittensor, et al., 2014). Recently, about 1,200 bird species are endangered with crisis of extinction in all organisms (Regan, et al., 2015). In mammals, somatic nuclear transfer is widely used for conserving endangered species, but it is hard to apply in avian species because of their physiological differences (Lanza, et al., 2000; Loi, et al., 2001; Moulavi, et al., 2017). In this circumstance, intra- or inter-specific germline chimeras have been considered as an alternative tool for bird conservation and restoration strategies (Kang, et al., 2008). Thus, germline competent stem cells, including primordial germ cell (PGC) and spermatogonial stem cell (SSC), have been studied as a tool for germline chimeric bird production for bird conservation in domestic fowls.

Quail (*Coturnix japonica*) has been studied as a suitable model for developmental biology, because it is easy to observe and manipulate quail embryos from all developmental stages (Selleck, 1996). Also quail has many advantages as an experimental animal model for bird conservation researches, due to its small body size, high egg production rate and short generation period. (Shin, et al., 2008; Nakamura, et al., 2013; Choi, et al., 2015; Jung, et al., 2017). In particular way, germ cell migration through blood stream enabled exogenous germ cell transplantation in bird species (Eyalgiladi, et al., 1981). There have been several studies reported about germline chimeric quail production through PGC transfer into recipients' embryos (Ono, et al., 1998; Kim, et al., 2005; Park, et al., 2008). The efficiency of germline

transmission was ranged from 1.8 to 63.0 % by non-cultured blood PGC (bPGC) (Ono, et al., 1998), 2.2 to 4.7 % by non-cultured gonadal PGC (gPGC) (Kim, et al., 2005) and 2.4 to 2.5 % by liquid nitrogen preserved gPGC (Chang, et al., 1998). However, because of varied germline transmission efficiency and insufficient donor cells, PGC mediated methods have limitations for practical applications. To overcome these limitations, there were several trials for establishing *in vitro* quail gPGC cultivation system and germline chimeric quail production. Although short term culture of quail gPGC were established, but it has also limitations for PGC survival duration and varied germline transmission efficiency (Park, et al., 2008; Yakhkeshi, et al., 2018). Therefore, it is necessary to develop alternative way for producing germline chimeric quails by different type of germline competent stem cells.

As an alternative germline competent stem cells for PGC, SSC could be also an important source for bird conservation researches (de Rooij, 2017). Originally, SSC have been suggested as a useful cell source for regenerative therapies for human, due to its unique characteristics, including ability to self-renewal and differentiating into mature gamete (Takashima and Shinohara, 2018). Testicular transplantation of SSC was firstly suggested at 1994 and actively studied in mammals such as mice and human (Brinster and Avarbock, 1994). Also there were several reports about SSC testicular transplantation in other mammals like goat and sheep (Zheng, et al., 2014). From these reports, donor derived progeny were successfully produced from transplanted SSCs. However, their efficiency was still low, because of low proportion of SSC in adult testis (Fayomi and Orwig, 2018).

Thus, enrichment and purification of SSC methods were should be studied to overcome limitations

Several studies for inducing enrichment and purification of SSC from adult testis by various manners have been reported. In mammals, differential plating (Giassetti, et al., 2016), density gradient centrifugation (Liu, et al., 2011; Panda, et al., 2011), and antibody-mediated purification methods, such as fluorescence activated cell sorting (FACS) (Shinohara, et al., 2000) and magnetic cell sorting (MACS) (Buageaw, et al., 2005) methods were well established. In quail, also differential plating of testicular cells for enriching SSC was reported previously, and even germline chimeric quails were produced (Pramod, et al., 2017; Kim, et al., 2018). However, there were also limitations for practical application, and enrichment of quail SSC is still in challenge. Furthermore, SSC specific antibody which is targeting surface protein on SSC-mediated purification methods were settled as a standard protocol in mammals. Representatively, ITGA6, ITGB1 and GFRA1 surface markers were widely used for mouse SSC purification. However, in case of quail, there are few studies about molecular characteristic in quail SSC and even no commercial antibodies for SSC specific surface markers. In this situation, we intended to applying density gradient centrifugation as an alternative method for enriching SSC in quail. Because SSC has relatively lower density compared to other mature germ cells and somatic cells in testis, density gradient centrifugation was widely used for enriching SSC in mammals (Goodyear and Brinster, 2017). There were no reports about density of quail SSC, so here we tried to verify density gradient centrifugation mediated SSC enrichment in quail. And density gradient

centrifugation could be an alternative and efficient way for germline chimeric bird production and even bird conservation studies.

2. Materials and methods

Animal management

Japanese quails (*Coturnix japonica*) were used for experiments. The Institute of Laboratory Animal Resources, Seoul National University (SNU-190401-1) approved animal care and experiments regarding to quails. Quails were managed as according to the standard management program at the University Animal Farm, Seoul National University (Pyeongchang, Korea). All procedures containing animal management, reproduction and surgical transplantations were governed by standard operating protocols.

Single cell isolation of testicular cells

Testes were surgically dissected from sexually matured quails (10-weeks-old) and washed several times with phosphate buffered saline (PBS) containing 1x antibiotic-antimycotic reagent (Gibco, Carlsbad, CA, USA). Tunica albuginea and connective tissues were removed on a Petri dish with Hank's Balanced Salt Solution (HBSS) containing 0.05 % trypsin-EDTA (Gibco). After mincing the testes, dissociated tissues were collected and incubated at 37 ℃ water bath for 20 min. Every ten minutes, pipetting was conducted by serological pipettes during incubation, and after finishing the incubation, trypsin EDTA solution was inactivated by same volume of Dulbecco's minimum essential medium (DMEM) containing 5 % fetal bovine serum (FBS). The cell suspension was harvested by centrifugation (1250 rpm, 5 min), and washed three times with PBS containing 1x antibiotic-antimycotic reagent. The final cell suspension was filtered through 40 μm nylon cell strainers (BD Falcon, Franklin Lakes, NJ, USA).

Density gradient centrifugation

Density gradient centrifugation of testicular cells was conducted with Ficoll-Paque PLUS (GE Healthcare Life Science, Chicaga, IL, USA), Percoll (Sigma Aldrich, St. Louis, MO, USA) and sucrose (Duchefa Biochemie, Haarlem, Netherland) solution. For constructing continuous Ficoll gradient, 5.7 mL of cell suspension in PBS was mixed with 4.3 mL of Ficoll in 15ml tube (Sarstedt, Nümbrecht, Germany). Percoll was diluted into 0, 20, 30, 40 and 50 % (volume / volume) with PBS, respectively. And sucrose was also diluted into 0, 20, 30, 40 and 50 % (weight / volume) with PBS, respectively. 2 mL of each diluted solution was cautiously transferred to 15 mL tube, and same volume of cell suspension in PBS was carefully layered on the top of the gradient. All tubes were then centrifuged at 800×g for 30 min at room temperature. After centrifugation, cells from differently layered fractions were harvested and washed three times with PBS containing antibiotic-antimycotic reagents.

Quantitative RT-PCR

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed to measure the expression of SSC-specific and pluripotency genes in mRNA level. Total RNA samples from whole testis, each fraction and QM7 cell line were prepared using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and complementary DNA (cDNA) library was synthesized from 1 μg of total RNA by reverse transcribing with Superscript III First-Strand Synthesis System (Invitrogen). The PCR mixture was prepared by adding 2 μL of cDNA, 2 μL of PCR buffer, 0.4 μL of 2.5 mM dNTP, 1 μL of 20 x Eva green, 2 μL of 10 pmol forward and reverse

primer (**Table 3-2**), 0.1 μL Taq DNA polymerase and 12.5 μL of ultrapure water in PCR tube. PCR condition was programed as initial incubation at 95 ℃ for 5min followed by 40 cycle at 95 ℃ for 30 s 60 ℃ for 30 s and 72 ℃ for 30 s. Continuous fluorescence measurement was conducted during melting curve program (increasing temperature from 55 ℃ to 95 ℃ at a rate of 0.5 ℃ per 10 s) after PCR, and PCR products are loaded on 1 % agarose gel for electrophoresis. Gene expression was quantified by the $2^{-\Delta\Delta Ct}$ method, and every gene's expression value was normalized to that of quail β-actin (*ACTB*). qRT-PCR analysis of gene expression in mRNA was performed by using CFX96 Real-Time PCR system (Bio-Rad, Hercules, CA, USA).

RNA probe hybridization

Probes for hybridization was synthesized from total RNA of testicular cells. cDNA was amplified by using primers shown in **Table 3-3**, and the PCR products were cloned with the pGEM-T Easy Vector System (Promega, Madison, WI, USA). After sequence verification, recombinant plasmid was amplified with T7 (5′-TGTAATACGACTCACTATAGGG-3′) and SP6 (5′-CTATTTAGGTGACAC TATAGAAT-3′) specific primers. PCR amplicons were extracted from agarose gel with Wizard® SV Gel and PCR Clean-Up System (Promega), and probe was synthesized by following general protocol of DIG RNA Labeling Kit (Roche, Basel, Switzerland). Hybridization sample preparation was performed by following the our standard operation protocol with modifications (Han, et al., 2018). Briefly, Cells separated by Ficoll were incubated at 4 % paraformaldehyde at 4 ℃ overnight for fixation. Fixed cells were dehydrated by methanol and washed with diethyl pyrocarbonate (DEPC)-PBS. After Post-fixation step, samples were incubated with

SSC-specific mRNA binding probes in hybridization solution at 68 ℃ for overnight. After washing, blocking and antibody incubating with anti Digoxigenin-AP Fab Fragment (Roche), cells were mounted on the slide glass and bright field image was obtained by inverted microscope (Nikon, Tokyo, Japan).

Transmission electron microscopy (TEM)

Cells separated by Ficoll were incubated at 4 ℃ in Karnovsky's Fixation solution for 4 h. After three times of washing with 0.05 M sodium cacodylate buffer, post fixation step was conducted at 4 ℃ by adding 2 % Osmium tetroxide and 0.1 M carcodylate buffer for 2 h. Briefly, after washing samples with distilled water, dehydration was followed with serial concentration of ethanol from 30 % to 100 %. In embedding step, ethanol was replaced with propylene oxide, and SPURR's resin was serially added by increasing its concentration to 100 %. Samples were incubated at 50 ℃ for overnight, and resin blocks were sectioned by Ultramicrotome (EM UC7, Leica, Wetzlar, Germany). All image was obtained by Transmission Electron Micro Scope 80kV (JEM1010, JEOL, Akishima, Japan).

Transplantation of SSC enriched cells

Procedure of enriched SSC transplantation was following as previous report (Kim, et al., 2018). Briefly, 40 mg/kg concentration of busulfan (Sigma Aldrich) dissolved in *N*,*N*-dimethyl formamide (Merck, Darmstadt, Germany) was injected intraperitoneally into 10-weeks-old quail recipients at three weeks before the transplantation. The cell suspensions (3×10^6 of whole testicular cells and enriched SSC labeled with PKH-26 in trypan blue diluted solution) were surgically

injected after anesthetizing the recipients with 10 mg/kg concentration of Zoletil (Virvac, Carros, France). After opening recipient quail's abdominal cavity with blades, surgical site was fixed with Weitlaner-Locktite Retractor (Fine Science Tools, Foster city, CA, USA), and simply injected at left testis. Surgical site was sutured with 4-0 coated VICRYL (Ethicon, Somerville, NJ, USA) from inner and outer skins.

Cryosection of recipient testis

One week after transplantation, recipient was sacrificed for detecting PKH-26 labeled cells in transplanted testis. Dissected testis was incubated in PBS containing 4 % paraformaldehyde for 16 h. After washing with PBS, testis was incubated in PBS containing 5 % sucrose for 30 min and 3h respectively, and testis was incubated in 30 % sucrose solution for 16 h. Sliced testis was frozen with O.C.T compound (Leica) on the liquid nitrogen. Sample was sectioned at freezing microtome (CM1860, Leica) by 10 μm thickness. Sectioned samples were fixed on the slide glass, and mounted with ProLong® Gold antifade reagent and 4′,6-diamidino-2-phenylindole (Invitrogen) for nucleus staining.

Testcross of germline chimeric quails

Whole testicular cell and enriched SSC transplanted recipient quails were recovered for up to one week in segregated cage under standard quail management program. Wild plumage (WP, d^+/d^+) and black plumage (D, *D/D*) recipients were mated with WP (d^+/d^+) and D (*D/D*) of female quails, respectively. From their progeny, hybrid type (D/d^+) quails were produced. Their genomic DNA was obtained from feather follicle cells and PCR analysis was performed by wild

plumage quail specific primer (WSP) and black quail specific primer (BSP) which are detecting MC1R gene SNPs between two types of quail (**Table 3-4**). PCR condition was programed as initial incubation at 95 ℃ for 5 min followed by 35 cycle at 95 ℃ for 30 s, 60 ℃ for 30 s and 72 ℃ for 60 s.

Statistical analysis

Relative expression of SSC-specific and pluripotency genes between upper and lower fractions separated by Ficoll, Percoll and sucrose mediated density gradient methods was analyzed by Student's t-test using GraphPad Prism statistical software (GraphPad Software, La Jolla, CA). And the values of SSC-specific and pluripotency gene expression in whole testicular cells and all upper fractions were subjected to ANOVA and Bonferroni's Multiple Comparison Test using GraphPad Prism statistical software. The germline transmission efficiency between whole testicular cell and enriched SSC transplanted group was also analyzed by Student's t-test using GraphPad Prism statistical software. In all statistical analysis, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ was considered significant.

3. Results

Density gradient centrifugation of quail testicular cells

At first, testes tissues were dissected from sexually mature quails with wild plumage (WP, d^+/d^+). Enzymatically single cell digested adult quail testicular cells were then separated by density gradient centrifugation. Three different types (Ficoll, Percoll and sucrose solution) of density gradient solutions were constructed for enriching SSC. After centrifugation, testicular cells were separated as largely two fractions in each gradient solutions. From the Ficoll gradient, the cell layer (Ficoll-1 fraction) was detectable at the top of the tube, and the cell pellet (Ficoll-2 fraction) was located at the bottom of the tube (**Figure 3-1A**). Similarly, in the Percoll gradient, the cell layer (Percoll-1 fraction) was located at the 20 % Percoll gradient, and the cell pellet (Percoll-2 fraction) was located at the bottom of the tube (**Figure 3-1B**). In addition, in the sucrose gradient, two cell layers were located at the 20 and 30 % sucrose gradient, respectively (**Figure 3-1C**). The morphology of the separated cells in each fractions were distinguishable between the Ficoll and Percoll fractions. The cells in the Ficoll-1 fraction exhibited round and conspicuous a cell size (approximately 20-25 μm of diameter), whereas cells in the Ficoll-2 fraction showed red blood cell-like and sperm-shaped irregular morphology. The relatively large and round cells containing cell debris and sperm-shaped cells are observed in the Percoll-1 fraction, but only red blood cell-like cells were observed in the Percoll-2 fraction. In the case of sucrose gradients, there was no significant morphological difference between the Sucrose-1 and Sucrose-2 fractions (**Figure 3-1D**).

Characterization of separated testicular cells

For characterizing cells in each fractions, we performed PCR analysis with SSC-specific (*GFRA1, ITGA6,* and *ITGB1*) and pluripotency (*POUV* and *NANOG*) gene targeting primers. All of SSC-specific and pluripotency markers were detected in upper fractions (Ficoll-1, Percoll-1 and Sucrose-1), but intensity of amplicon was variable (**Figure 3-2A**). Next, we analyzed expression level of SSC-specific and pluripotency genes in each fractions by qRT PCR. As a result, relative expressions of SSC-specific and pluripotency markers were significantly higher in the Ficoll-1 fraction compared to those in the Ficoll-2 fraction. These expression patterns were also shown between the Percoll-1 and Percoll-2 fractions, but relative expressions of SSC-specific and pluripotency markers between the Sucrose-1 and Sucrose-2 fractions showed different patterns from those of the Ficoll- and Percoll-mediated separations. The relative expressions of the *GFRA1*, *NANOG*, and *POUV* genes were significantly higher in the Sucrose-1 fraction compared to those in the Sucrose-2 fraction, but there were no significant differences in the expression of the *ITGA6* and *ITGB1* genes (**Figure 3-2B**). For comparing relative mRNA expressions of SSC-specific and pluripotency markers between upper fractioned cells, we subsequently performed qRT-PCR even with whole testicular cells. The Sucrose-1 fraction showed similar expression of the *ITGA6, NANOG* and *POUV* genes with whole testicular cells. The Percoll-1 fraction showed significantly higher expression of *ITGA6*, *NANOG* and *POUV* compared to Sucrose-1 fractions but showed similar (*ITGB1*) or significantly lower (*GFRA1*) expression in some genes. The Ficoll-1 fraction showed significantly higher expression of the *GFRA1*, *ITGB1*, *NANOG* and *POUV* genes among the other groups but showed similar expression of *ITGA6* in the

Percoll-1 fraction (**Figure 3-2C**). To visualize SSC-specific RNA expression, we first tried immunocytochemistry (ICC) with commercially available antibodies targeting *GFRA1*, *ITGA6*, or *ITGB1*, which were suitable for mouse, rat and human proteins. However, there were no positive signals with these specific antibodies in all the experimental groups (**data not shown**). Then, we performed RNA probe hybridization for visualizing SSC-specific mRNA expressions in the Ficoll-1 and Ficoll-2 fractions. The expressions of SSC-specific marker genes were detectable at the Ficoll-1 fraction, but there were no signals at the Ficoll-2 fraction (**Figure 3-3A**). Next, we analyzed the ultrastructural characteristics of cells in the Ficoll-1 and Ficoll-2 fractions. There was no heterochromatin, which is abundant in differentiated or differentiating spermatogonial cells, in the nucleus of the Ficoll-1 fraction cells while, electron-dense heterochromatin was found in the nucleus of the Ficoll-2 fraction cells (**Figure 3-3B**). Collectively, we concluded that SSC could be mostly enriched by Ficoll mediated density gradient centrifugation.

Production of germline chimeric quails

Subsequent transplantation of SSC enriched cells and whole testicular cells (as a control) was performed. Cells with PKH-26 fluorescent staining in trypan blue solutions were transplanted in busulfan treated recipient's left testis. The transplanted testis was dissected one-week after transplantation (**Figure 3-4A**), and it showed that the donor cell solution was successfully injected into the seminiferous tubules with trypan blue (**Figure 3-4B**). And cryosection results revealed that there were no signals in right testis (**Figure 3-4C**), but red fluorescent stained donor cells were detectable on left testis seminiferous tubules (**Figure 3-4D**). Even genomic

DNA analysis revealed that donor *D*-specific amplicon (Black quail specific primers; BSP) was detected by PCR in the left testis of recipient and mature sperm, but not in right testis of recipient (**Figure 3-4E**). Next, testcross analysis was performed to evaluate germline transmission feasibility and efficiency. Phenotypically, the hybrids (D/d^+) exhibited dark brown feathers, whereas the WP line (d^+/d^+) derived progenies had yellow and black stripes, and *D* line (*D/D*)-derived progenies had all black feathers (**Figure 3-4F**). Their genomic DNA was analyzed by breed-specific primers, and both amplicons were detected in genomic DNA of phenotypically hybrid type progeny (**Figure 3-4G**). Then we compared germline transmission efficiencies between whole testicular cells and cells in Ficoll-1 fraction transplanted group. In the control group, donor-derived progeny production rate ranged from 0 % to 4.4 % (3/68), and the average was 1.4 ± 1.4 %. On the other hand, the Ficoll-1 fraction group ranged from 0 % to 13.2 % (7/53) of donor-derived progeny production rate, and the average was 8.4 ± 1.7 % (**Figure 3-4H and Table 3-1**). In conclusion, germline transmission efficiency of the Ficoll-1 fraction group was significantly ($P<0.05$) enhanced compared with that of whole testicular cell transplanted group.

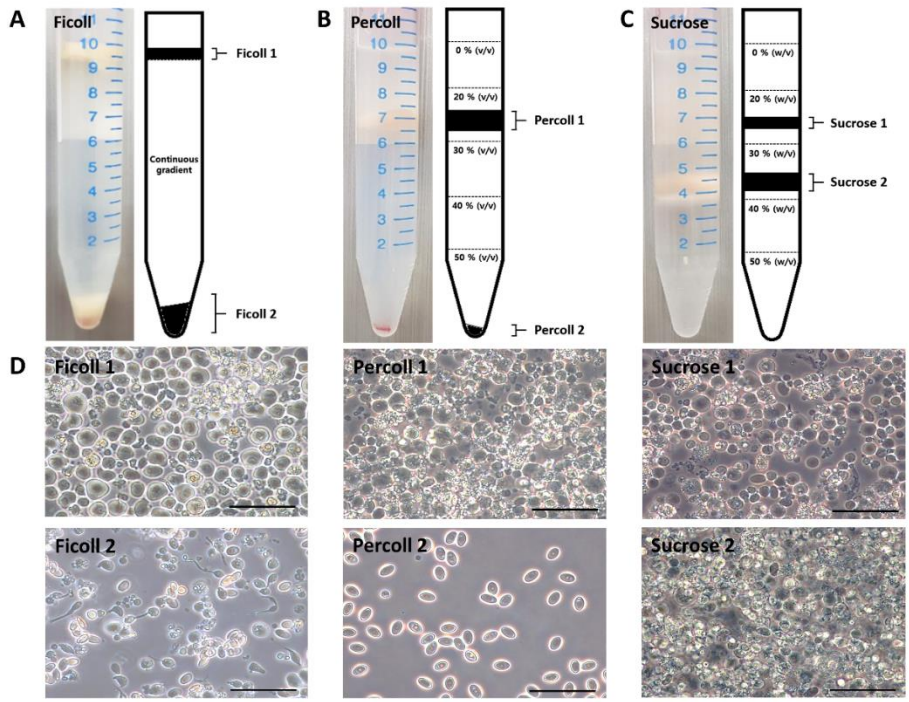

Figure 3-1. Density gradient centrifugation of adult quail testicular cells. Separated adult quail testicular cells by (A) Ficoll, (B) Percoll and (C) sucrose density gradient centrifugation. Percentages of solutions were indicated as volume/volume in Percoll and weight/volume in sucrose gradients, respectively. Each fraction was named after its gradient and numbered. (D) Bright field image of cells harvested from each density gradient. Scale bar = 100 µm.

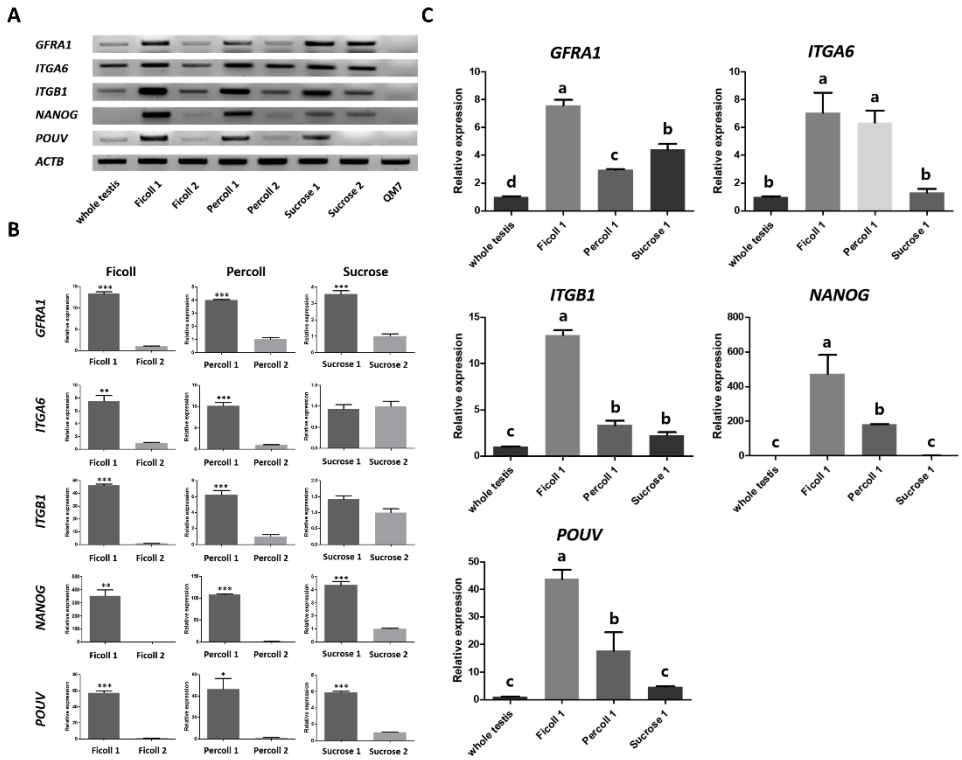

Figure 3-2. Characterization of density gradient separated testicular cells. (A) Amplicons of SSC-specific (*GFRA1*, *ITGA6*, and *ITGB1*) and pluripotency (*POUV* and *NANOG*) genes from the total RNA of whole testis, each fraction and QM7 cell line. (B) Expression level of the genes between upper (Ficoll-1, Percoll-1, Sucrose-1) and lower (Ficoll-2, Percoll-2, Sucrose-2) fractions in Ficoll, Percoll and sucrose gradients, respectively. (C) Relative expression level of each gene from the Ficoll-1, Percoll-1, Sucrose-1 fractions and whole testis. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ was considered significant and different letters (a-d) represented significant differences at $P < 0.05$.

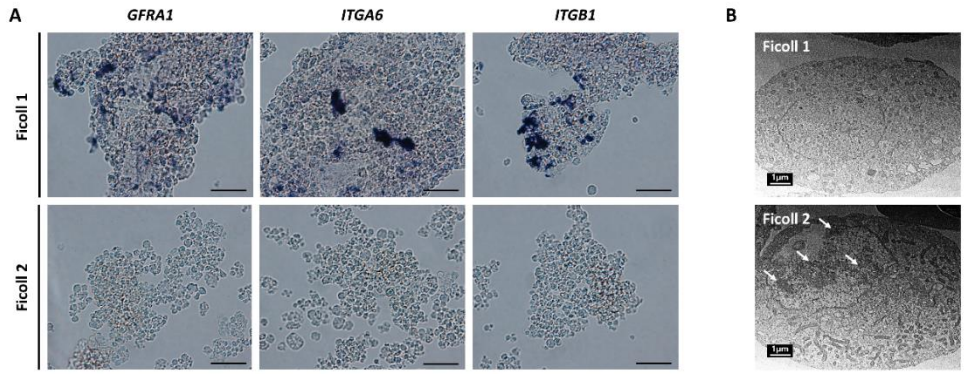

Figure 3-3. Verification of enriched SSC in Ficoll-1 fraction. (A) RNA probe hybridization in Ficoll-1 and Ficoll-2 fractions with SSC-specific (*GFRA1*, *ITGA6* and *ITGB1*) markers. Scale bar = 100 µm. (B) Transmission electron microscopy (TEM) image from the Ficoll-1 and Ficoll-2 fractions. White arrows indicate electron-dense heterochromatin in nucleus. Scale bar = 1 µm.

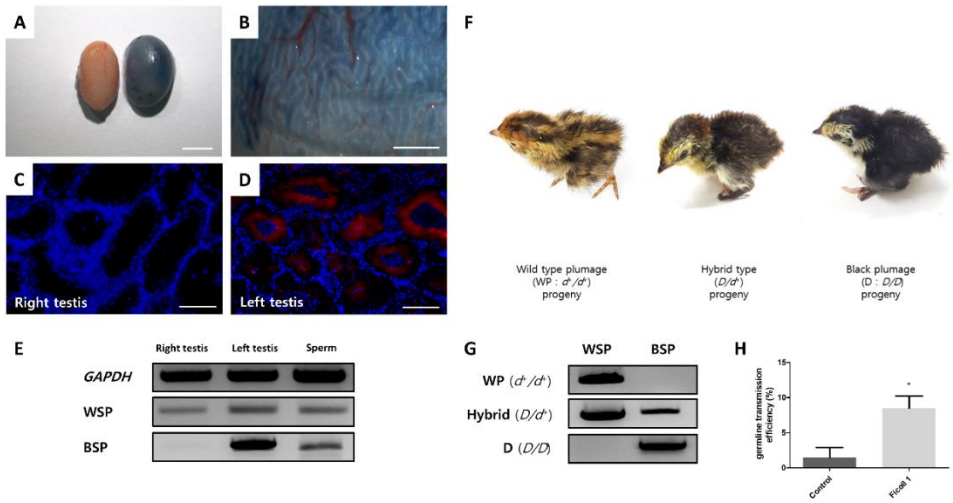

Figure 3-4. Enhancing germline transmission efficiency by SSC enriched fraction transplantation. (A) Bright field image of right testis and Trypan blue stained left testis from recipient quail. Scale bar = 5 mm. (B) Magnified image of transplanted left testis. Scale bar = 1 mm. Sectional view of (C) right and (D) left testis. Scale bar = 100 µm. (E) Genomic DNA analysis in right, left testes and sperm of recipient quail with *GAPDH* and breed specific primers. (F) Phenotype of WP (d^+/d^+), hybrid and representative D (*D/D*) type progeny, and (G) their genomic DNA analysis results. (H) Germline transmission efficiency between control and Ficoll-1 fraction cells transplanted groups. * $P < 0.05$ was considered significant.

Table 3-1. Birth of donor-derived progeny from germline chimeric quail

Type of donor cells	Recipient ID	No. of transplanted cells	No. of hatched quails	No. (%) of hybrid type (D/d^+) quails
whole testicular cells*	Control #1	3×10^6	68	3 (4.4)
	Control #2	3×10^6	27	0 (0.0)
	Control #3	3×10^6	32	0 (0.0)
Subtotal	**Control group**		127	3 (2.3)
Ficoll-1 fraction cells†	Ficoll-1 fraction #1	3×10^6	17	1 (5.8)
	Ficoll-1 fraction #2	3×10^6	43	5 (11.6)
	Ficoll-1 fraction #3	3×10^6	71	9 (12.6)
	Ficoll-1 fraction #4	3×10^6	51	0 (0.0)
	Ficoll-1 fraction #5	3×10^6	53	7 (13.2)
	Ficoll-1 fraction #6	3×10^6	55	5 (9.1)
	Ficoll-1 fraction #7	3×10^6	28	2 (7.1)
Subtotal	**Ficoll-1 fraction group**		318	29 (9.1)

* Single cell dissociated adult quail testicular cells

† Upper fraction of adult quail testicular cells separated by Ficoll density gradient

Table 3-2. Primer information used for qRT-PCR analysis

Serial No.	Gene	Primer sequence(5'-3')	Source
1	q*GFRA1*	F : CTGCTCCTGTCGAGACGTAG R : GGAGGCAGTCAGCGTAGTTC	NM_205102
2	q*ITGA6*	F : GTTGGTGATGAGAGCCTCCG R : CCGCTATGGTTGGCTCTTGG	XM_015868494.1
3	q*ITGB1*	F : GGGGACCAGATTGGATGGAG R : CCAGGTGACATTTCCCATCA	NM_001323203.1
4	q*NANOG*	F : TGCACACCAGGCTTACAGCAGTG R : TGCTGGGTGTTGCAGCTTGTTC	XM_015871944.1
5	q*POUV*	F : GCTGGAGAGCTTCTTCCGCA R : GACTCGTTGCCAAAGGGCAG	XM_015878605.1
6	q*ACTB*	F : GGGTGTTGGTAACAGTCCGG R : AGGAGATCACAGCCCTGGCA	XM_015876619.1

Table 3-3. Primer information used for RNA probe synthesis

Serial No.	Gene	Primer sequence(5'-3')	Source
1	q*GFRA1*	F : ACTTGGCTCTGCCCTTAGCG R : GGCAGTCAGCGTAGTTCTCC	NM_205102
2	q*ITGA6*	F : AGCCTCTTCGGCTTCTCGCT R : GCTCCAGTGAAAGCGCTCTCTG	XM_015868494.1
3	q*ITGB1*	F : GGAGAACCTAACAGCCCTGC R : AATTCCAGCAACCACGCCGG	NM_001323203.1

Table 3-4. Primer information used for testcross analysis

Serial No.	Gene	Primer sequence(5'-3')	Source
1	WSP*	F : CGTCAGCAACCTGGCCG R : GTCTGTGCTGCTGCCTACCA	MC1R gene from wild plumage quail
2	BSP†	F : GCGTCAGCAACCTGGCCA R : GTCTGTGCTGCTGCCTACCA	MC1R gene from black plumage quail
3	*GAPDH*	F : GGGAAGTTGTGGAGGGATGG R : GGTTGGCACACGGAAAGCCA	NC_029516.1

* Wild plumage quail specific primer

† Black plumage quail specific primer

4. Discussion

Previously, we reported studies of isolation, characterization and *in vitro* cultivation of SSC from chicken and quail, and we successfully produced SSC-mediated germline chimeric birds (Lee, et al., 2006b; Jung, et al., 2007; Pramod, et al., 2017; Kim, et al., 2018). In this study, we showed the successful enrichment of quail SSC using density gradient centrifugation and the enhancement of germline transmission through the enriched SSC transplantation into testes. This strategy could be an alternative method for enriching the SSC population from quails without specific antibodies or an *in vitro* culture system.

SSC is distinct germline competent stem cell in adult testis tissue with self-renewing properties and continuous differentiating ability. SSC was studied mainly in mammals, including rodents, domestic animals and even humans, for male fertility studies (Kossack, et al., 2009; Kanatsu-Shinohara, et al., 2011; Zheng, et al., 2014). In vertebrate species, SSC commonly has unique characteristics compared to other type of spermatogonia in morphological, molecular and ultrastructural aspects. Due to their uniqueness, enrichment and purification methods for SSC in several species were actively studied and well established (Shinohara and Brinster, 2000; Rodriguez-Sosa, et al., 2006; Kokkinaki, et al., 2011; de Barros, et al., 2012; Binsila, et al., 2018; Garbuzov, et al., 2018).

Morphologically, it was reported that SSC has a relatively round shape, clear cytoplasm and distinctive larger size compared to other somatic cells in mouse testis (Kokkinaki, et al., 2009). From our results, the cells in the Ficoll-1 fraction

also showed round shape, clear cytoplasm and a diameter of 20-25 μm. And these kinds of features were similar to typical SSC morphology in other species. Furthermore, SSC usually has lower density compared to other spermatogonial and somatic cells because of their relative large size (Goodyear and Brinster, 2017). Thus, many researchers tried to apply density gradients for enriching SSC in various species. In this study, discontinuous density gradients were constructed at the desired densities, except Ficoll. Because of its isotonic characteristic, Ficoll density gradients were usually constructed by continuous manner. Therefore, the continuous density gradient of Ficoll was constructed by mixing with PBS. As expected, cells whose densities were lower than others were separated from whole testicular cells and located in the upper fractions in all density gradients. And we assumed that SSC has relatively lower density than other testicular cells not only in mammals but also in birds.

SSC also has distinct molecular characteristics on their surfaces. Thus, SSC specific surface marker-mediated purification methods were actively studied in mammals. Firstly, *ITAG6* and *ITGB1*, one of the laminin receptors, were reported as a surface marker for SSC purification. And *ITGA6*- and *ITGB1*- mediated selection showed 8.4 and 3.8 fold enrichments of SSC (Shinohara, et al., 1999). Next, *GFRA1* was suggested as a powerful SSC specific marker according to knockout studies which demonstrated that glial cell line-derived neurotrophic factor (*GDNF*) and its receptor *GFRA1* are indispensable for SSC self-renewal (Meng, et al., 2000). In addition, the embryonic stem cell marker (*CD9*), epithelial cell adhesion molecule (*EPCAM*), melanoma cell adhesion molecule (*MCAM*) and their combination were

used as SSC purification markers, and exhibited a maximum 560.5-fold higher enrichment of functional SSC (Kanatsu-Shinohara, et al., 2004; Ryu, et al., 2004; Kanatsu-Shinohara, et al., 2012). The non-surface stem cell marker, *OCT4* was also used for SSC purification by FACS-based isolation and transplantation methods using *OCT4*-GFP conjugated transgenic animal resource (Ohmura, et al., 2004). We assumed that mRNA expression levels of SSC-specific and pluripotency genes could be a useful parameter for confirming the degree of SSC enrichment from each fraction. As expected, the SSC was enriched at the upper fractions of all gradients, but the efficiency of the enrichment differed from each fractions. After comparing the relative expressions of SSC-specific and pluripotency markers in all upper fractions and whole testis by qRT-PCR, we concluded that SSC was concentrated mostly by Ficoll-mediated separation. However, it was difficult to use surface markers as an SSC purification tool because there were no commercially available antibodies and transgenic animals for SSC purification in quail. In this circumstance, we applied density gradient centrifugation for SSC enrichment, which has great advantages of simplicity and application possibilities for various avian species.

SSC experiences dynamic changes as they differentiate from type A spermatogonia to mature sperm and, following these nuclear changes, reorganization of heterochromatin is also accompanied during spermatogenesis. According to ultrastructural studies, heterochromatin distribution in type A spermatogonia was less frequently detected than other differentiated spermatogonial cells in mouse (Maezawa, et al., 2018). SSC also showed lower electron dense of heterochromatin and heterochromatin reaches maximum electron dense at late stage Type B

spermatogonia following spermatogenesis in fish (Lacerda, et al., 2014). Generally, the more differentiated cells in a particular lineage, the more heterochromatin could be found in the nucleus (de Rooij and Russell, 2000). According to this studies, we confirmed that there was no heterochromatin in cell from the Ficoll-1 fraction, while electron-dense heterochromatin was detected in cell from the Ficoll-2 fraction. Collectively, these observations indicate that that quail SSC could be enriched through Ficoll density gradient centrifugation.

Spermatogonial transplantation methods for producing germline chimeric animals were well established in mammals. Because of outside located testes, it is easy to access and manipulate adult testis in mammals. Initially, whole testicular cell transplantation into mouse testes showed quite low donor-derived offspring production efficiency (0.8 %, 1/122), and it was enhanced as 15.3 % (6/39), 40 % (6/15) in each experimental group with busulfan treatment before transplantation (Brinster and Avarbock, 1994). At present, mouse SSC could be isolated with high purity by various positive (*ITGA6*, *ITGB1*, *THY-1*, *GFRA1*, and *CDH1*) and negative (*ITGA5*, *c-KIT*, *MHC-1*, and *CD45*) markers on surface (Phillips, et al., 2010). Moreover, an *in vitro* culture of SSC has been established, and the germline transmission efficiency reached over 70 % through transferring these cells into busulfan-treated recipient testes (Kanatsu-Shinohara, et al., 2016).

On the other hand, the application of these established methods directly in avian systems is still limited due to lack of commercially available antibodies and even in vitro culture system. Nevertheless, there have been several efforts for producing germline chimeric birds using SSC transplantation. Transplantation of

juvenile (4-week-old) and adult (24-week-old) chicken testicular cells resulted about 7.8 % (5/64) of germline chimeric chicken production rate. However, the donor-derived offspring production rate was very low (less than 1 %) (Lee, et al., 2006a). Next, gamma irradiation was used to produce infertile chickens and to enhance the germline chimeric bird production efficiency. As a result, approximately 20 % (2/9, 3/15) of recipients recovered their fertility (Trefil, et al., 2006). In case of quail, transplantation of 14-day cultured SSC into busulfan treated recipients resulted 0 % (0/64) to 16.7 % (7/42) efficiency of donor-derived offspring production (Kim, et al., 2018). In this study, germline chimeric quails, which were transplanted with enriched SSC, showed 0 % (0/51) to 13.2 % (7/53) of donor-derived offspring production efficiency. Our results showed similar germline transmission efficiency with the transplantation of *in vitro* cultured SSC, but it is simple and much easier methods compared with previous methods.

The simplicity of the Ficoll-mediated separation methods enables easier acquisition of SSC and the practical application of germline chimera production for the restoration of endangered birds. Even there were studies about interspecific transplantation of SSC between mouse and rat (Zhang, et al., 2003; Shinohara, et al., 2006). And there were also reports about transplantation of quail and pheasant testicular cells into a vitelline vein of a chicken embryo and they resulted in gonadal migration of donor cells (Roe, et al., 2013; Kim, et al., 2014). According to these studies, we can expect to produce interspecific germline chimera with more enhanced efficiency and thus contribute to restoration of endangered birds.

In conclusion, we successfully enriched SSC from quail testis through Ficoll-mediated density gradient centrifugation and produced germline chimeric quails. This simple and enhanced strategy is expected to be highly applicable for the conservation of avian species.

CHAPTER 4

IN VITRO GENOME MODIFICATION OF QUAIL GERMLINE COMPETENT STEM CELLS BY ADENOVIRAL VECTOR MEDIATED CRISPR/CAS9 DELIVERY

1. Introduction

Germline competent stem cells, which contain primordial germ cells (PGCs) and spermatogonial stem cells (SSCs), are the only animal cells that can transfer entire genetic information to the next generation. They are able to self-renew and differentiate into mature gametes. These characteristics make germline competent stem cells a suitable source for generating transgenic animals. Thus, understanding the origin and related processes of various germline competent stem cells is crucial for practical applications. PGCs, which are the precursors of mature sperms and eggs, are the most actively studied germline competent stem cells in avian species, especially in chickens. Vick et al. (1993) were the first to report avian leucosis virus- and spleen necrosis virus-mediated gene transfer into chicken PGCs. Later, lentiviruses were introduced into purified chicken PGCs to produce transgenic chicken cell lines (Motono, et al., 2010). The long-term *in vitro* cultivation of chicken PGCs and cell lines have been established for transgenic studies (Macdonald, et al., 2010; Park and Han, 2012; Lee, et al., 2016). However, there are only few studies regarding PGC manipulation in avian species such as the Japanese quail (*Coturnix japonica*), and long-term, *in vitro* culture systems have not been established. Only lentivirus-infected, PGC-mediated transgenic studies have been reported in quail (Shin, et al., 2008; Kwon, et al., 2010). SSCs, which are the precursors for spermatogenesis, comprise a distinct germ line of competent stem cells in adult testis. SSCs have been successfully isolated and cultured in mammalian species including mice and humans (Nagano, et al., 2003; Kossack, et al., 2009). Although it is difficult to access SSCs because testis tissues are located in inner body in avian species, several reports have detail the characterization and cultivation of

SSCs in such species, including chickens and quail (Jung, et al., 2007; Pramod, et al., 2017). However, there are certain limitations in the practical application of producing SSC-mediated transgenic avian cell lines. Thus, further investigations regarding PGCs and SSCs for *in vitro* culture and obtaining basic data for inducing genome edition in germline competent stem cells in quail are required.

Transcription activator-like effector nuclease and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) systems are some common genome editing platforms. The CRISPR/Cas9 system is widely favored owing to its efficiency and simplicity compared with other platforms. Moreover, this system has successfully showed feasibility in chicken somatic cells and germ cells (Abu-Bonsrah, et al., 2016; Oishi, et al., 2016). In quail, there are reports regarding CRISPR/Cas9-mediated genome modification in quail somatic cells (Ahn, et al., 2017; Kim, et al., 2017b) and even production of genome edited animal by blastodermal injection of adenoviral vector (Lee, et al., 2019). However, there are no reports regarding CRIPSR/Cas9-mediated genome edition in *in vitro* cultured germline competent stem cells.

Viral vectors have been used for introducing genetic material into living organisms or cells. Several studies have reported on adenoviral and adeno-associated *in vivo* or *in vitro* viral vector-mediated gene transfer into male germline stem cells of mice and pigs (Takehashi, et al., 2007; Honaramooz, et al., 2008; Zeng, et al., 2013; Watanabe, et al., 2017). Owing to their ability to deliver genetic materials into a host, viral vectors have been used as a tool for gene therapy (Lundstrom, 2018).

Each viral vector has distinct characteristics, allowing it to be modified and advanced for this purpose. The integration of the Cas9 protein can induce off-target effects in germline cells, which may result in abnormal germ-cell differentiation. Adenoviral and adeno-associated viral vectors are the representative non-integrating viral vectors.

Quail is considered as an appropriate model for developmental studies because of their small body size, low maintenance cost, and short generation period. In addition, it is easy to manipulate its eggs during all stages of embryonic development (Selleck, 1996), allowing quail to be a suitable model organism for transgenic research. This allows for *in ovo* imaging of embryos with reporter genes during egg incubation period (Sato, et al., 2010). For generating transgenic animals, pronuclear injection and somatic cell nuclear transfer have been identified as the most successful strategies. However, unlike mammals, it is difficult to produce transgenic animals by embryo transfer or nuclear transfer methods in avian systems because of their yolk and large cytoplasm of oocytes (Lee, et al., 2013). Under these circumstances, germline competent stem cell-mediated production of germline chimera may be regarded as an alternative method for producing transgenic avian cell lines (Han, et al., 2015; Han and Park, 2018). Recently, a blastodermal injection of adenoviral vectors was utilized to produce transgenic quail (Lee, et al., 2019), suggesting the feasibility of adenoviral transduction into quail germline competent stem cells.

In this study, we selected non-integrating viral vectors for delivering the CRISPR/Cas9 system into quail germline competent stem cells. We introduced an adenoviral vector in *in vitro*, cultured quail and chicken germline competent stem cells for more efficient application. We investigated the feasibility of CRISPR/Cas9 transfer into avian germline competent stem cells and identified genome modification and its efficiency *in vitro*.

2. Materials and methods

Animal management

White leghorn chickens (*Gallus gallus*) and Japanese quails (*Coturnix japonica*) were used for experiments. The Institute of Laboratory Animal Resources, Seoul National University (SNU-190401-1) approved animal care and experiments regarding to quails. Animals were managed as according to the standard management program at the University Animal Farm, Seoul National University (Pyeongchang, Korea). All procedures containing animal management, reproduction and surgical transplantations were governed by standard operating protocols.

Gonadal cell preparation

Embryonic gonads were dissected from Hamburger and Hamilton stage 28 of quail and chicken embryos. Embryonic gonadal tissues were physically dissociated in HBSS containing 0.05 % trypsin-EDTA by pipetting. Dissociated tissues were incubated at 37 ℃ water bath for 20 min. Every 10 minutes, pipetting was conducted, and after finishing the incubation, trypsin EDTA solution was inactivated by same volume of Dulbecco's minimum essential medium (DMEM) containing 5 % fetal bovine serum (FBS). The cell suspension was harvested by centrifugation (1250 rpm, 5 min), and washed three times with PBS containing 1x antibiotic-antimycotic reagent. The final cell suspension was filtered through 40 μm nylon cell strainers (BD Falcon, Franklin Lakes, NJ, USA). Single cell dissociated cells were shortly cultured in knockout DMEM with 10 % of fetal bovine serum (FBS), 2 % of chicken serum, 2 mM of L-glutamine, 1 mM of sodium pyruvate, 0.1

mM of NEAA, 1x antibiotic-antimycotic reagent, 0.1 mM of mercaptoethanol, 1x nucleoside and 10 ng/mL of human bFGF.

Primary SSC preparation

Testes were surgically dissected from sexually matured adult quail and chicken. After removal of tunica albuginea, testes were minced with blades on the petridish containing HBSS with 0.05 % trypsin EDTA. After mincing the testes, dissociated tissues were collected and incubated at 37 ℃ water bath for 20 min. Every 10 minutes, pipetting was conducted by serological pipettes during incubation, and after finishing the incubation, trypsin EDTA solution was inactivated by same volume of Dulbecco's minimum essential medium (DMEM) containing 5 % fetal bovine serum (FBS). The cell suspension was harvested by centrifugation (1250 rpm, 5 min), and washed three times with PBS containing 1x antibiotic-antimycotic reagent. The final cell suspension was filtered through 40 μm nylon cell strainers (BD Falcon, Franklin Lakes, NJ, USA). Density gradient centrifugation of testicular cells was conducted with Ficoll-Paque PLUS (GE Healthcare Life Science, Chicaga, IL, USA). For constructing continuous Ficoll gradient, 5.7 mL of cell suspension in PBS was mixed with 4.3 mL of Ficoll in 15ml tube (Sarstedt, Nümbrecht, Germany). Tubes were then centrifuged at 800×g for 30 min at room temperature. After centrifugation, cells from upper layered fractions were harvested and washed three times with PBS containing antibiotic-antimycotic reagents. Enriched SSC was shortly culture in PGC media with adding 15 ng/mL of GDNF.

Viral vector transduction

QM7 and DF-1 cell lines and primary cells were transduced with MOI of 100 viral vectors (Vigene Bioscience) day after seeding cells into the 12-well culture plate. Because all viral vectors containing GFP coding sequence, GFP signals were observed one, three and five days after transduction from each cells. For optimizing adenoviral transduction, poly-L-lysine (Sigma Aldrich) was added with serial increase of concentration. All images were obtained by inverted microscope (Nickon). Adenoviral vector infected cells were harvested and their transduction efficiency and viability were analyzed by fluorescence-activated cell sorting (FACS) analysis and trypan blue assay.

Magnetic-activated cell sorting (MACS)

Adenoviral vector infected gonadal cells were harvested three days after transduction. One million quail and chicken gonadal cells were labeled with anti-QCR1 mouse immunoglobulin (Ig) G isotype and anti-SSEA1 mouse Ig M for 20 min at a room temperature in buffer solution, respectively. The cells were then incubated in 80 μL of buffer solution with 20 μL of secondary antibody with micro-magnetic beads (Miltenyi Biotec) for 15 min at 4 ℃. After incubation, 500 μL of buffer solution was carefully added and PGC purification was conducted in magnetic field. Eluted MACS-positive and negative cells were washed with PBS.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA samples from all adenoviral vector infected cells were prepared using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and complementary

DNA (cDNA) library was synthesized from 1 µg of total RNA by reverse transcribing with Superscript III First-Strand Synthesis System (Invitrogen). The PCR mixture was prepared by adding 2 µL of cDNA, 2 µL of PCR buffer, 0.4 µL of 2.5 mM dNTP, 1 µL of 20 x Eva green, 2 µL of 10 pmol forward and reverse primer (**Table 4-1**), 0.1 µL Taq DNA polymerase and 12.5 µL of ultrapure water in PCR tube. PCR condition was programed as initial incubation at 95 ℃ for 5min followed by 35 cycle at 95 ℃ for 30 s 60 ℃ for 30 s and 72 ℃ for 30 s.

Immunocytochemistry (ICC)

All adenoviral vector infected cells were fixed in 4 % paraformaldehyde for 15 min. After washing with PBS, blocking was performed in PBS containing 5 % goat serum and 1 % bovine serum albumin (BSA). For *DAZL* protein staining, cells were incubated in 0.01% of Triton-X 100 for 20 min in room temperature additionally. Samples were then incubated overnight at 4 ℃ with primary antibodies (*QCR1*, *SSEA1* and *DAZL*). After washing three times in PBS, samples were incubated in secondary antibody conjugated with phycoerythrin (PE) for 1h at room temperature. After washing, samples were mounted with ProLong Gold antifade reagent and visualized under fluorescence microscope.

Vector construction

Three different single guide RNA sequences targeting for *transferrin* and *Hoxb13* gene were recruited with consideration of targeting site and PAM sequence (**Table 4-2**). We synthesized sense and antisense oligonucleotides (Bionics) and carried out annealing using 30s at 95 ℃, 2min at 72 ℃, 2min at 37 ℃ and 2min at

25 ℃ thermal cycle. Synthesized oligonucleotides were inserted in PX459 plasmid backbone using 5 min at 37 ℃ and 10 min at 16 ℃ for 15 thermal cycle with BbsI and buffer addition. Constructed vectors were transfected into QM7 cell line with lipofectamine (Thermo scientific) by corporation's guiding protocol.

T7E1 assay

Genomic DNA of transfected cells was extracted. Genomic regions encompassing the CRISPR/Cas9 target sites were amplified using specific primer sets (**Table 4-3**). PCR analysis of targeted site was performed in total volume of 20 µL containing 100 ng of genomic DNA, 10 x PCR buffer, 0.4 µL of dNTPs, 10 pmol of forward and reverse primers and 0.5 U Taq polymerase (BioFACT) under following 5 min at 95 ℃, 35 cycles of 30 s at 95 ℃, 30 s at 60 ℃, 30 s at 72 ℃ and final 5 min at 72 ℃ thermal cycles. PCR amplicons were re-anealed to form heteroduplex DNA structure after denaturation. Subsequently, the heteroduplex amplicons were treated with 5 units T7E1 endonuclease (New England Biolabs) for 20 min at 37 ℃. All treated and non-treated PCR amplicons were analyzed by 1 % agarose gel electrophoresis.

Sequencing analysis

Target sized ampilcons were extracted by Wizard SV Gel and PCR Clean-up System (Promega). 3 µL of PCR products were incubated in total volume of 10 µL containing 2 x rapid ligation buffer, 1 µL of T easy vector (Promega) and 1 µL of T4 DNA ligase at room temperature for 3 h. PCR amplicons were ligated with T easy vector, and the ligated plasmids were up incubated on ice for 30 min

with competent E.coli. E.coli and plasmid mixture was heat shocked at 42 ℃ for 30 sec, and incubated in shaker with LB broth (duchefa) for 1 h. The mixture was spread on the ampicillin (Sigma) and x-gal (Bioneer) added Agar LB plate (Duchefa), and incubated at 37 ℃ for overnight incubation. White colony was collected respectively and incubated in shaker with ampicillin added LB broth for 16 h. E.coli was harvested, and plasmid DNA was extracted by mini-prep kit (Favorgen) by manufacturer's protocol. Finally, plasmid was analyzed from M13R binding site by sequencing service (Bionics).

3. Results

Introducing a non-integrating, viral vector system into quail and chicken cells

To confirm the transfection availability of integration-free viral vectors in quail and chicken cells, we performed *in vitro* viral transduction using an MOI of 100 for each viral vector. All integration-free viral vectors had green fluorescent protein (GFP) coding sequences with a CMV promoter. Prepared primary gonadal cells, SSCs, and somatic cell lines were transduced by adenoviral- and adeno-associated viral vector serotypes (AAV1, AAV2, AAV5, AAV6, AAV7, AAV8, and AAV9). From primary quail gonadal cells and SSCs, only the adenoviral vector-transduced group showed GFP-positive signals. In QM7 cell lines, AAV2-, AAV5-, and AAV7-transduced groups showed GFP-positive signals (**Figure 4-1**). Chicken cells showed a different pattern of viral transduction. There were no positive signals in primary gonadal cells and SSCs, Moreover, only the adenoviral vector-transduced group showed GFP-positive signals in DF-1 cell line (**Figure 4-2**). HEK 293 cell line was used as a positive control. Although there were differences in GFP intensity and transduction efficiency in HEK 293 cells, all viral vector-treated groups showed GFP-positive signals (**Figure 4-1**).

Optimization of adenoviral vector transduction in quail cells

To optimize the transduction of adenoviral vectors in quail cells, we added poly-L-lysine in serially increasing concentrations (0–20 μg/mL). In all quail cells, transduction was primarily enhanced following the addition of 1 μg/mL of poly-L-lysine (**Figure 4-3A**). The percentages of GFP-positive cells in each experimental group were measured using FACS analysis. Adenoviral transduction

efficiencies were optimized by adding 1 µg/mL of poly-L-lysine in quail gonadal cells (30.6%), primary SSCs (55.3%), and QM7 cell lines (78.9 %) (**Figure 4-3B**). The viability of these cells was measured using trypan blue assay, and the viability of each cell reduced as poly-L-lysine was added. However, the viability increased to >80% following the addition of 1-µg/mL poly-L-lysine (**Fig 3C**). Conversely, the same transduction condition showed a different pattern of GFP percentage in chicken cells, except the DF1 cell line (**Figure 4-4A**). Chicken gonadal cells and primary SSCs showed 0.11% and 0.22% of GFP-positive cells following the addition of 1-µg/mL poly-L-lysine (**Figure 4-4B**). The percentage of GFP-positive cells in all experimental groups of chicken gonadal cells and primary SSCs was low. Similarly, the viability of quail and chicken cells decreased as the concentration of poly-L-lysine increased (**Figure 4-4C**).

Characterization of adenoviral vector-transduced germline competent stem cells

Adenoviral vector-transduced quail gonadal cells were sorted using anti-QCR1 monoclonal antibody under optimized conditions. Immunocytochemistry results revealed that QCR1-positive cells still expressed GFP signals and that GFP-positive cells were present in the QCR1-negative group (**Fig 5A**). We analyzed the mRNA expression of germline competent stem cell-related markers (VASA, DAZL, NANOG, POUV, GFRA1, ITGA6, and ITGB1) using RT-PCR. While quail primary SSCs expressed all germline competent stem cell-related markers, QCR1-positive quail PGCs expressed all genes except GFRA1, which is a specific marker for adult SSCs. The expression of germline competent stem cell-related markers was not

observed in QM7 cells (**Figure 4-5B**). In particular, immunocytochemstry results revealed that *DAZL*-positive cells still expressed GFP signals in quail PGCs and SSCs. There were no *DAZL*-positive signals in GFP-positive QM7 cells (**Figure 4-5C**), even when the same procedures were performed in chicken cells using anti-SSEA1 monoclonal antibodies (**Figure 4-6A**). We confirmed *SSEA1*-mediated sorting using immunocytochemistry and performed RT-PCR to characterize adenoviral vector-transduced chicken cells. Similar to quail cells, chicken primary SSCs expressed all germline competent stem cell markers; however, *GFRA1* was not expressed in chicken PGCs (**Figure 4-6B**). *DAZL* staining revealed that there were no GFP-positive cells in chicken PGCs and SSCs. Similar to QM7 cells, there were no *DAZL*-positive signals in GFP-positive DF1 cells (**Figure 4-6C**).

Selection of a single guide RNA sequence for targeted mutagenesis in QM7 cell line

To select a single guide RNA sequence inserted into adenoviral vectors targeting for *Transferrin* and *Hoxb13*, synthesized oligonucleotides were inserted into a PX459 plasmid. Based on a previous study, a single guide RNA sequence targeting *Transferrin* was selected as 50% of the targeted mutation efficiency (Kim, 2018). In addition, we designed a single guide RNA sequence targeting *Hoxb13*. Ligated vectors were transfected into QM7 cells, and their genomic DNA was analyzed using T7E1 assay and sequencing. Sequencing results showed that the efficiencies of the targeted mutation of guide RNAs were 66.6% for q*Transferrin* and 75% for q*Hoxb13*, respectively (**Figure 4-7**).

Adenoviral vector mediated targeted mutagenesis in quail germline competent stem cells

Adenoviral vector was designed as delivering CRISPR/Cas9 system conjugated with EGFP by 2A self-cleaving peptide sequence and controlled by CBh promoter (**Figure 4-8A**). Quail PGC, SSC and QM7 cell line were transduced by adenoviral vector containing CRISPR/Cas9 with previously optimized condition (**Figure 4-8B**). Because each vectors contain GFP coding sequences conjugated by 2A self-cleaving peptides, we could identify approximate transduction efficiency by observing GFP signals. Each targeting knockout vectors were well transfected into quail primary PGC, SSC and QM7 cells *in vitro*. One, three and five days after transduction QM7 cells, primary PGC and SSC were harvested respectively. T7E1 assay and sequencing analysis were performed from genomic DNA of the harvested cells. T7E1 assay and sequencing analysis showed about 33.3 % of target gene mutation efficiencies in quail primary PGC and SSC. At the same time, target gene mutation efficiency was measured as about 77.8 % in QM7 cells (**Figure 4-8C**).

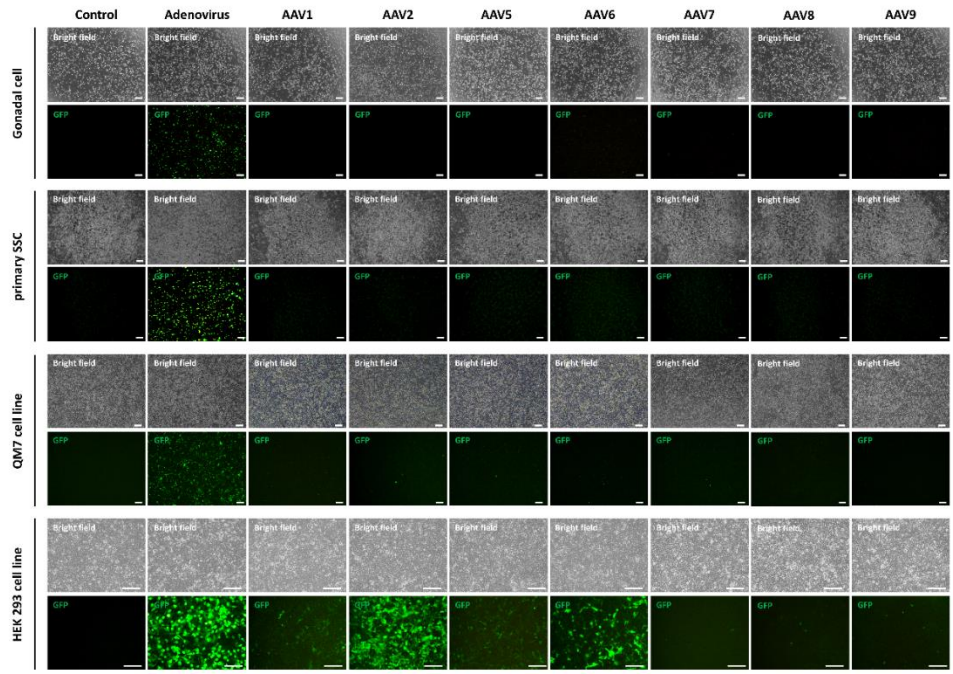

Figure 4-1. ***In vitro*** **non-integrating viral vector transduction in primary quail germline cells, somatic cell lines and HEK 293 cell lines.** Adenoviral vector and adeno-associated viral vector transduction with MOI of 100. Three day after transduction, GFP positive cells were observed from adenoviral vector treated quail embryonic gonadal cells. Five days after transduction, GFP positive cells were observed from adenoviral vector treated enriched quail SSC. One day after transduction, GFP positive cells were observed from adenoviral vector, AAV serotype 2, 5, 6 and 7 treated QM7 cell line. One day after transduction, GFP positive cells were observed from all type of non-integrating viral vector treated HEK 293 cell line. Scale bar = 200 µm.

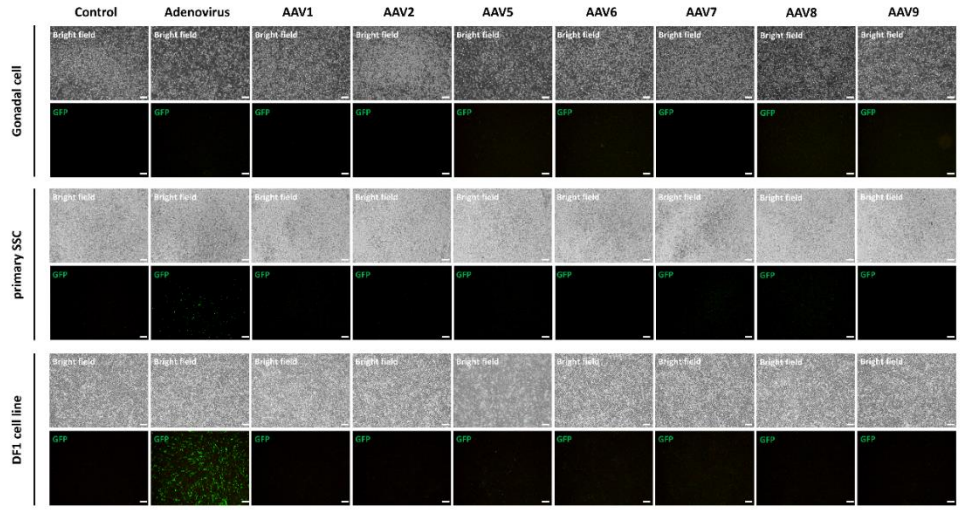

Figure 4-2. ***In vitro*** **non-integrating viral vector transduction in primary chicken germline cells and somatic cell lines.** Adenoviral vector and adeno-associated viral vector transduction with MOI of 100. Five day after transduction, GFP positive cells were observed from adenoviral vector treated enriched chicken SSC. One day after transduction, GFP positive cells were observed from adenoviral vector treated DF-1 cell line. Scale bar = 200 µm.

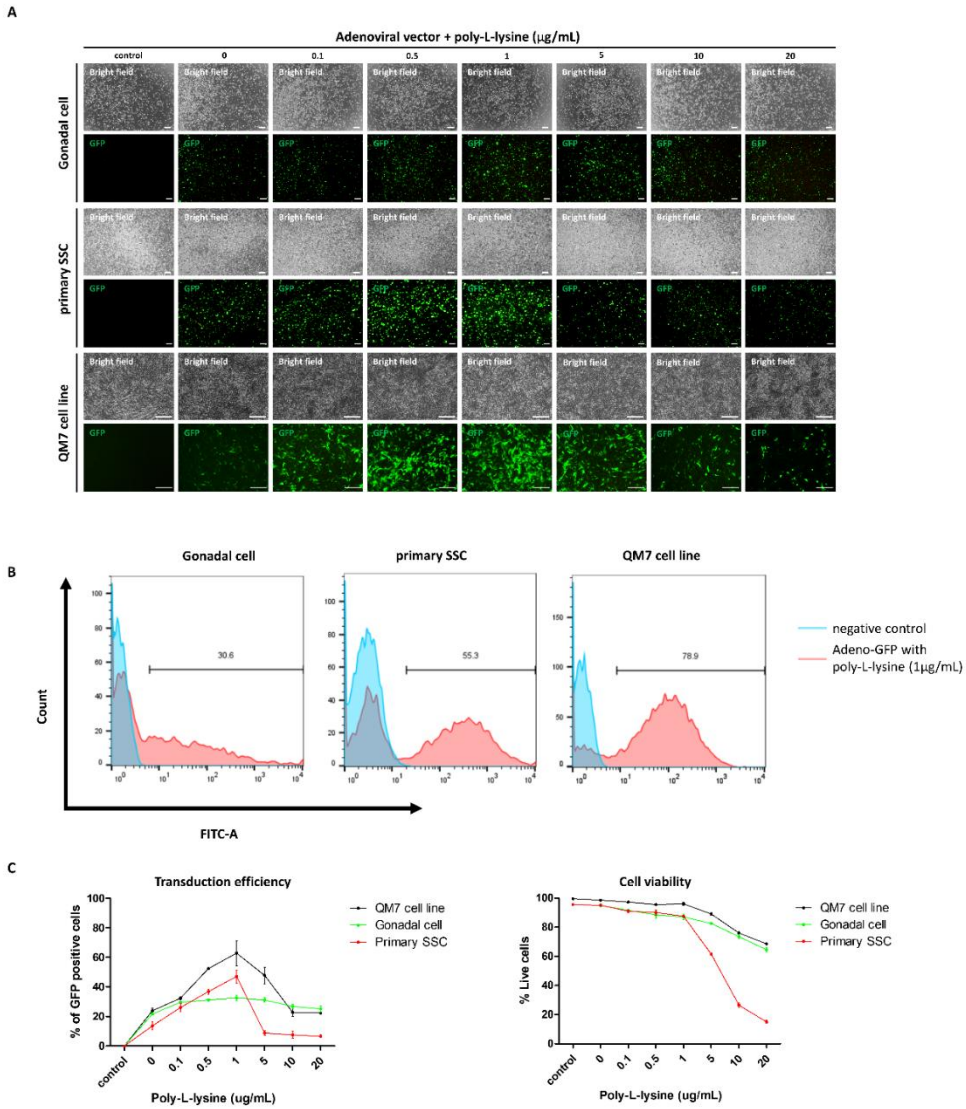

Figure 4-3. Optimization of *in vitro* adenoviral transduction in quail cells with poly-L-lysine. (A) GFP positive signals were observed three, five and one day after adenoviral transduction with adding poly-L-lysine from quail primary gonadal cells, primary SSC and QM7 cell line. (B) FACS analysis was performed 1 μg/mL concentration of poly-L-lysine added group. (C) Proportion of GFP positive cells were measured by FACS analysis, and cell viability was measured by trypan blue assay. Scale bar = 200 μm.

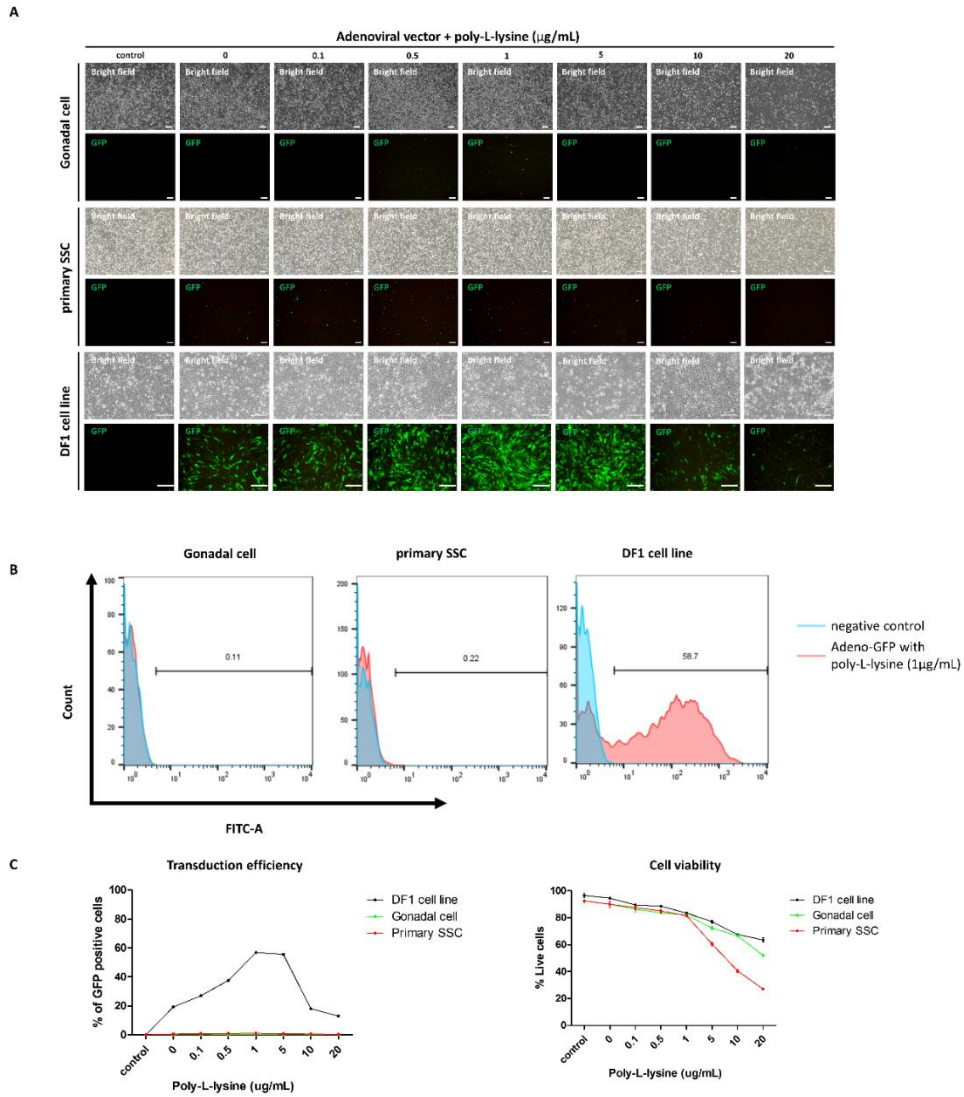

Figure 4-4. Optimization of *in vitro* adenoviral transduction in chicken cells with poly-L-lysine. (A) GFP positive signals were observed three, five and one day after adenoviral transduction with adding poly-L-lysine from chicken primary gonadal cells, primary SSC and DF-1 cell line. (B) FACS analysis was performed 1 μg/mL concentration of poly-L-lysine added group. (C) Proportion of GFP positive cells were measured by FACS analysis, and cell viability was measured by trypan blue assay. Scale bar = 200 μm.

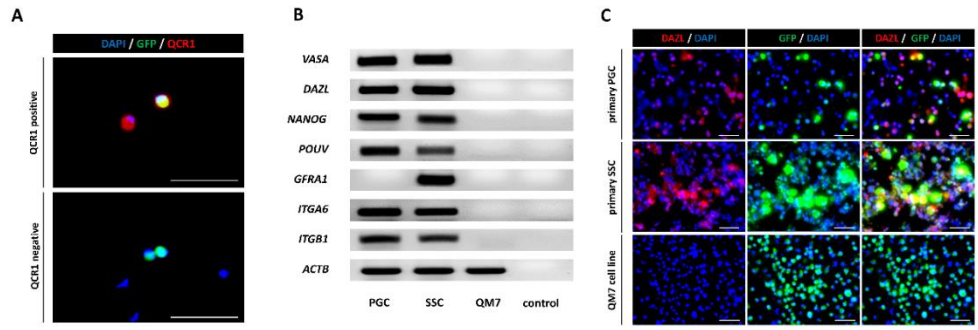

Figure 4-5. Characterization of adenoviral vector transduced quail germ cells. (A) Quail PGC was isolated by MACS with QCR1 marker, and QCR1 positive and negative cells were immunostained with anti-QCR1 mAb. (B) mRNA expression of germ cell, pluripotency and germline stem cell specific markers were analyzed from quail PGC, SSC and QM7 cell line by PCR. (C) Immunostaining of aednoviral vector transduced quail cells with anti-DAZL mAb. Scale bar = 50 μm.

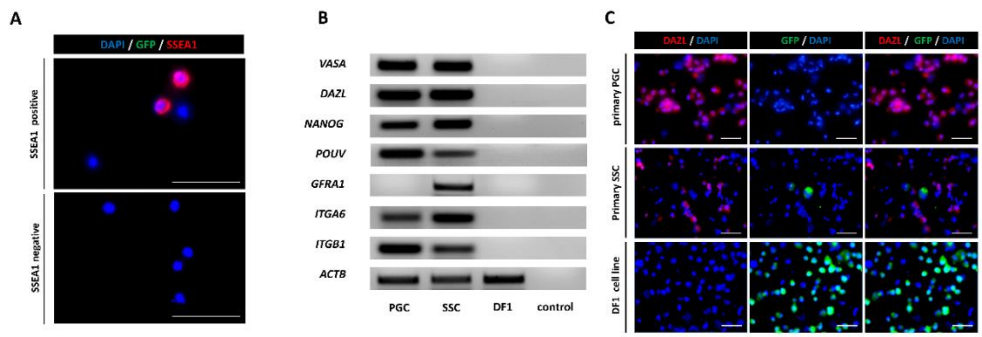

Figure 4-6. Characterization of adenoviral vector transduced chicken germ cells. (A) Chicken PGC was isolated by MACS with SSEA1 marker, and SSEA1 positive and negative cells were immunostained with anti-SSEA1 mAb. (B) mRNA expression of germ cell, pluripotency and germline stem cell specific markers were analyzed from quail PGC, SSC and QM7 cell line by PCR. (C) Immunostaining of adenoviral vector transduced quail cells with anti-DAZL mAb. Scale bar = 50 μm.

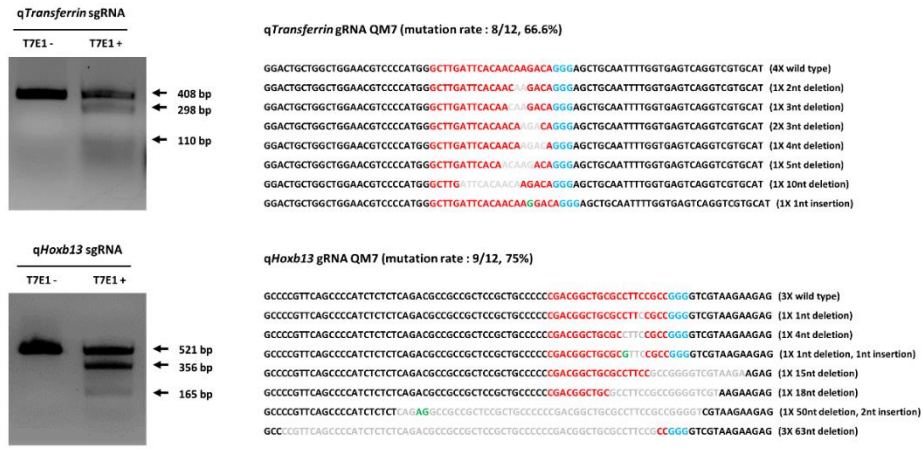

Figure 4-7. Verification of targeted mutagenesis efficiency of selected single guide RNA in QM7 cell line. CRISPR/Cas9 and single guide RNA targeting quail genes (*Transferrin*, *Hoxb13*) were delivered by PX459 constructed vector into QM7 cell line. T7E1 assay and sequencing analysis of transfected QM7 cells.

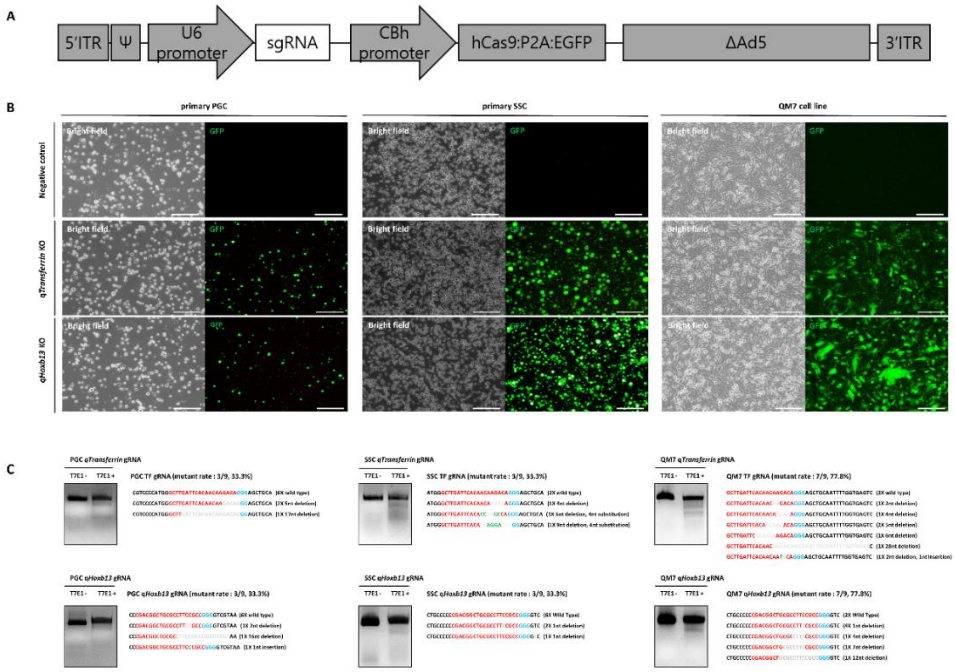

Figure 4-8. ***In vitro*** **genome modification in quail germline competent stem cells and somatic cell line by delivering CRISPR/Cas9 system with adenoviral vector.** (A) Map of constructed adenoviral vector. (B) CRISPR/Cas9 delivery with adenoviral vector into quail primary PGC, SSC and QM7 cell line. (C) T7E1 assay and sequencing analysis of adenoviral transduced quail cells. Scale bar = 100 μm.

Table 4-1. Primer information used for RT-PCR analysis

Serial No.	Gene	Primer sequence(5'-3')	Source
1	q*DAZL*	F : CAACTATCAGGCTCCACCAC R : CTCAGACGGTTTTCAGGGTT	XM_015853881.1
2	q*VASA*	F : CCTTGCAGCCTTTCTTTGTC R : CCTTGCAGCCTTTCTTTGTC	XM_015848726.1
3	q*GFRA1*	F : CTGCTCCTGTCGAGACGTAG R : GGAGGCAGTCAGCGTAGTTC	NM_205102
4	q*ITGA6*	F : GTTGGTGATGAGAGCCTCCG R : CCGCTATGGTTGGCTCTTGG	XM_015868494.1
5	q*ITGB1*	F : GGGGACCAGATTGGATGGAG R : CCAGGTGACATTTCCCATCA	NM_001323203.1
6	q*NANOG*	F : TGCACACCAGGCTTACAGCAGTG R : TGCTGGGTGTTGCAGCTTGTTC	XM_015871944.1
7	q*POUV*	F : GCTGGAGAGCTTCTTCCGCA R : GACTCGTTGCCAAAGGGCAG	XM_015878605.1
8	c*DAZL*	F : TCCCAGAGCCCACACAGATG R : AAGTGATGCGCCCTCCTCTC	XM_015281256.2
9	c*VASA*	F : TGACTTATGTCCCCCCTCCT R : GTAATGGTGCTGGAGGGTCA	NM_204708.2
10	*c*GFRA1	F : ACTTGGCTCTGCCCTTAGCG R : GGCAGTCAGCGTAGTTCTCC	NM_205102.1
11	c*ITGA6*	F : ACTTGGCTCTGCCCTTAGCG R : GGCAGTCAGCGTAGTTCTCC	NM_205289.1
12	c*ITGB1*	F : AGCCTCTTCGGCTTCTCGCT R : GCTCCAGTGAAAGCGCTCTCTG	XM_015281260.2
13	c*NANOG*	F : CAGCAGACCTCTCCTTGACC R : TTCCTTGTCCCACTCTCACC	NM_001146142.1
14	c*POUV*	F : GTTGTCCGGGTCTGGTTCT R : GTGGAAAGGTGGCATGTAGAC	NM_001309372.1
15	*ACTB*	F : GGGTGTTGGTAACAGTCCGG R : AGGAGATCACAGCCCTGGCA	XM_015876619.1

Table 4-2. Primer information used for CRISPR/Cas9 expression vectors

Serial No.	Gene	Primer sequence(5'-3')	Source
1	q*Transferrin*	F : caccgGCTTGATTCACAACAAGACA R : aaacCGAACTAAGTGTTGTTCTGT	XM_01587 1233.1
2	q*Hoxb13*	F : caccgCGACGGCTGCGCCTTCCGCC R : aaacGCTGCCGACGCGGAAGGCGG	XM_01588 5566.1

Table 4-3. Primer information used for T7E1 analysis

Serial No.	Gene	Primer sequence(5'-3')	Source
1	q*Transferrin*	F : GTTGTGAAGAAAGGCAGCGG R : CTTGGGGAACGCTTTTTGGG	XM_0158712 33.1
2	q*Hoxb13*	F : CAGTGGAAGTAGATAGGGAGACT R : CCCCCCATTTGCTGATGGGT	XM_0158855 66.1

4. Discussion

In this study, we introduced the CRISPR/Cas9 system into quail germline competent stem cells with an adenoviral vector. When preparing PGCs from early embryonic stages, specific surface markers have been suggested in both chicken and quail cells. SSEA1 was used for isolating chicken PGCs from embryonic gonads (Mozdziak, et al., 2005), and PGCs purified using QCR1-mediated, magnetic-activated cell sorting from embryonic gonads resulted in a 50% germline transmission efficiency (Park, et al., 2008). Based on previous studies, we applied magnetic-activated cell sorting with SSEA1 and QCR1 monoclonal antibodies for purifying PGCs from adenoviral vector-transduced embryonic gonadal cells. SSCs were also prepared using the same methods along with Ficoll density gradient centrifugation. Using SSC's relatively low density, we simply enriched chicken and quail SSCs from whole testicular cells.

Due to non-integrating characteristics, adeno and adeno-associated viral vectors are usually preferred for *in vivo* gene therapy (Ehrke-Schulz, et al., 2017; Lau and Suh, 2017). However, these unique characteristics matched with the CRISPR/Cas9 system in primary germline competent stem cells. In addition, several studies have reported the delivery of the CRISPR/Cas9 system with adenovirus in mammalian cells including mouse, pig, and human cells (Maggio, et al., 2014; Wang, et al., 2015; Li, et al., 2018; Gao, et al., 2019). Here, we revealed that genome editing may be induced by adenoviral vector-mediated CRISPR/Cas9 system delivery in quail primary PGCs and SSCs.

Adenoviral vectors, which are non-enveloped, double-stranded DNA viruses with a packaging capacity of 35 kb, have been widely used as a gene delivery tool in biotechnology. More than 50 different types of adenoviral vectors exist in nature, and serotype 2- and 5-derived recombinant adenoviral vectors are commonly used for this purpose. We transduced serotype 5-derived adenoviral vector, with GFP coding sequences regulated by the CMV promoter, in quail primary embryonic gonads enriched with SSC and QM7 cell lines. Although there were differences in transduction efficiencies among quail cells, all cell types showed GFP-positive signals at 5 days after transduction *in vitro*. Similar experiment was performed with chicken cells, and adenoviral vector-transduced chicken primary SSCs and DF-1 cell lines showed GFP-positive signals at 5 days after transduction. However, there were no such signals in adenoviral vector-transduced chicken PGCs.

Most adenovirus serotypes use the coxsackie/adenovirus receptor (CAR) as the primary binding receptor (Zhang and Bergelson, 2005). CAR is an immunoglobulin superfamily member with two extracellular domains, working as a homotypic cell adhesion molecule and as an intracellular tight junction in various types of cells (Honda, et al., 2000). CAR-mediated adenoviral transduction is well defined in mammalian cells. The crystal structure of adenovirus fiber binds to CAR's N-terminal domain, which induces the entrance of the selected viral particle (Freimuth, et al., 1999). Even the mutation of fiber proteins on the adenovirus can block the binding of viral particles to the cell surface, resulting in adenoviral resistance (Zabner, et al., 1997). Similarly,

integrin has been recognized as important entry receptor. RGD peptides within a penton base can interact with several cellular integrins, which are members of a heterodimeric adhesion receptor family (Zhang and Bergelson, 2005). The engagement of integrins with penton bases can induce viral entrance activating signals, including Rho family GTPases and phosphoinositide-3-OH kinase (Li, et al., 1998a; Li, et al., 1998b). These procedures allow viruses to escape from the endosome. In addition, the mutation of RGD sequences reduces and slows viral internalization and infection (Bai, et al., 1993). In conclusion, both CAR and integrin receptors are simultaneously required for efficient adenoviral transduction; moreover, the distribution of receptors may differ for different types of cells and even species. Thus, we can assume that these receptors are absent in chicken primary gonadal cells and SSCs.

Polymers including polybrene, poly-L-lysine, and polyethyleneimine have been widely used for enhancing adenoviral transduction. Polymers increase the binding affinity of cell surface to viral particles. Several studies have shown that polymer-enhanced adenoviral transduction occurs in both mouse and human cells (Kasman, et al., 2009; Zhao, et al., 2014; Buo, et al., 2016). However, high concentrations of polymers can induce cell toxicity. Therefore, we optimized adenoviral transduction by adding poly-L-lysine, and 1 μg/mL poly-L-lysine showed the most optimal transduction efficiency and cell viability.

In this study, we constructed adeno CRISPR vectors using single guide RNA targeting *Transferrin* and *Hoxb13* in quail. We performed knockout

only for *in vitro* cells; however, targeted knockout for quail could provide essential information regarding embryonic development. Ovotransferrin is a glycoprotein that accounts for approximately 13% of egg albumen (Wu and Acero-Lopez, 2012). It functions as an antimicrobial agent and transports iron to the developing embryo (Giansanti, et al., 2012). For bioreactor models, an excessive amount of egg white protein may hinder the purification of biofunctional proteins. Thus, using an egg with controlled egg white composition can help in efficiently purifying a small amount of recombinant proteins.

Hoxb13 is related to the regression of the caudal spinal cord and tail vertebrae. In embryonic stage, somitogenesis actively proceeds during tail extension; however, as retinoic acid signal increases, *Hoxb13* is also activated and inhibits *Cyp26a*, *Wnt3a*, and Notch signaling for tail growth (Rashid, et al., 2014). In *Hoxb13*-knockout mice, an over growth of tail vertebrae has been reported (Economides, et al., 2003). A model with these features has more value in studies of avian species because it is related to evolutionary biology studies. Present birds have pygostyle tails, in which the skeleton has the shape of fused caudal vertebrae that form a single ossification. However, ancient birds displayed relatively longer tails compared with modern birds (Gatesy and Dial, 1996). We believe that this avian model can provide more information and advantages for future paleontological studies.

In summary, we introduced the CRISPR/Cas9 system using an adenoviral vector into quail germline competent stem cells. We optimized adenoviral transduction by adding poly-L-lysine, which showed approximately 33.3% of genomic DNA mutation in quail PGCs and SSCs. The findings of this study could be used as a guide for generating transgenic quail models for bioreactor and developmental studies.

CHAPTER 5

GENERAL DISCUSSION

Avian species have distinct characteristic different from mammals which their embryos are growing outside of the mother's body. Because of this characteristic, it is easy to observe and manipulate avian embryos. And It makes avian species as excellent developmental biology studying model. In addition, due to their short generation, high egg production rate avian species also considered as a good bioreactor model. Especially, chicken has been studied as a valuable bioreactor, disease resistance and human disease model, and actively studied for generating germline chimeric and transgenic chicken. These studies were mainly accomplished by investigating germline competent stem cells in chicken especially, primordial germ cells. Although there are many reported in vitro long-term culture methods of chicken primordial germ cells, but there were few studies reported about germline competent stem cells in other avian species like turkey, pheasant and quail. Investigators tried *in vitro* culture of primordial germ cells form the other avian species, but it showed limitation as short maintaining duration, and low ranged germline transmission efficiencies. Thus it is needed to develop alternative methods for generating germline chimeric and transgenic birds. In this regard, here, we demonstrated enrichment of spermatogonial stem cells by Ficoll density gradient solution and analyzed its feasibility and germline transmission ability by testicular transplantation and testcross analysis in quail. Finally, we introduced CRISPR/Cas9 system with adenoviral vector into quail germline competent stem cells, including primordial germ cells and spermatogonial stem cells, and analyzed their genomic DNA mutagenesis efficiency. It is expected beneficial basic studies for generating germline chimeric and transgenic quail and even in other avian species.

In first study, we enriched quail spermatogonial stem cells and performed transplantation and testcross analysis. Previously, we reported isolation, characterization and in vitro cultivation of chicken and quail spermatogonial stem cells. Also we tried producing spermatogonial transplantation mediated germline chimeric chicken and quail, but it showed low germline transmission efficiency in chicken, and varied germline transmission in quail. In this study, we showed the successful enrichment of quail Spermatogonial stem cell (SSC) using density gradient centrifugation and the enhancement of germline transmission through the enriched SSC transplantation into testes. From our results, the cells in the Ficoll-1 fraction also showed round shape, clear cytoplasm and a diameter of 20-25 μm. And these kinds of features were similar to typical SSC morphology in other species. Also we assumed that mRNA expression levels of SSC-specific and pluripotency genes could be a useful parameter for confirming the degree of SSC enrichment from each fraction. As expected, the SSC was enriched at the upper fractions of all gradients, but the efficiency of the enrichment differed from each fractions. After comparing the relative expressions of SSC-specific and pluripotency markers in all upper fractions and whole testis by qRT-PCR, we concluded that SSC was concentrated mostly by Ficoll-mediated separation. However, it was difficult to use surface markers as an SSC purification tool because there were no commercially available antibodies and transgenic animals for SSC purification in quail. In this circumstance, we applied density gradient centrifugation for SSC enrichment, which has great advantages of simplicity and application possibilities for various avian species. Finally, we confirmed that there was no heterochromatin, which is normally observed from differentiated of differentiating spermatogonial cells, at cells from the

Ficoll-1 fraction, while electron-dense heterochromatin was detected in cell from the Ficoll-2 fraction. Collectively, these results indicate that that quail SSC could be enriched through Ficoll density gradient centrifugation. And subsequent transplantation experiments resulted that enriched spermatogonial stem cell transplanted germline chimeric quail produced donor-derived progeny with 8.4 ± 1.7 % efficiency while that of control group was 1.4 ± 1.4 %. Our results showed similar germline transmission efficiency with the transplantation of *in vitro* cultured SSC, but it is simple and much easier methods compared with previous methods. Accordingly, the simplicity of the Ficoll-mediated separation methods enables easier acquisition of SSC and the practical application of germline chimera production for the restoration of endangered birds.

In second study, we introduced CRISPR/Cas9 system into quail germline competent stem cells with adenoviral vector and induced DNA mutagenesis. There were several studies about isolating germline competent cells in chicken and quail, and according to these previous studies, we also applied magnetic-activated cell sorting with SSEA1 and QCR1 monoclonal antibodies for purifying primordial germ cell (PGC) from adenoviral vector transduced embryonic gonadal cells. SSC was also prepared with same methods by Ficoll density gradient centrifugation. By using SSC's relatively low density, we simply enriched chicken and quail SSC from whole testicular cells. In addition, we tried introduce transgenes with non-integrating viral vectors including adeno and adeno-associated viral vectors into quail and chicken germline competent stem cells. As a results, adenoviral vector transduced cells showed the most positive transgene signals among the all serotypes of adeno- and

adeno-associated viral vector transduced quail cells including embryonic gonadal cells, enriched spermatogonial cells and QM7 cell line. However, different to quail cells, there were relatively low positive transgene signals in adenoviral vector transduced chicken primary SSC, even there was no signal in adenoviral vector transduced chicken embryonic gonadal cells. Most of adenovirus serotypes are using coxsackie/adenovirus receptor (CAR) as a primary binding receptor, and simultaneously, integrin was also recognized as important entry receptors for secondary penton base binding. both CAR and integrin receptors are simultaneously needed for efficient adenoviral transduction and distribution of receptors could be differ from types of cells and even species. Thus, we can assume that these kinds of receptors could lack or absent from primary gonadal cell and SSC in chicken. For enhancing adenoviral transduction, we introduced polymer, which helps cell to viral particle binding. Polymers including polybrene (pB), poly-L-lysine (pLL) and polyethyleneimine (pEI) were widely used for enhancing adenoviral transduction. However, high concentration of polymers can induce cell toxicity. Therefore, we optimized adenoviral transduction with addition of poly-L-lysine, and 1 μg/mL concentration of poly-L-lysine addition showed the most optimal transduction efficiency and cell viability. Finally, we constructed adenoviral vector delivering CRISPR/Cas9 with single guide RNA targeting for quail *Transferrin* and *Hoxb13* genes. With optimized conditions of adenoviral transduction, it showed approximately 33.3 % of genomic DNA mutation in quail PGC and SSC. In conclusion, our study could be used as a basic data for generating bioreactor and developmental studying model transgenic quails.

REFERENCES

Abu-Bonsrah, K. D., D. Zhang, and D. F. Newgreen. 2016. CRISPR/Cas9 Targets Chicken Embryonic Somatic Cells In Vitro and In Vivo and generates Phenotypic Abnormalities. Sci Rep 6:34524.

Ahn, J., J. Lee, J. Y. Park, K. B. Oh, S. Hwang, C. W. Lee, and K. Lee. 2017. Targeted genome editing in a quail cell line using a customized CRISPR/Cas9 system. Poult Sci 96:1445-1450.

Aponte, P. M., M. P. van Bragt, D. G. de Rooij, and A. M. van Pelt. 2005. Spermatogonial stem cells: characteristics and experimental possibilities. APMIS 113:727-742.

Bai, M., B. Harfe, and P. Freimuth. 1993. Mutations that alter an Arg-Gly-Asp (RGD) sequence in the adenovirus type 2 penton base protein abolish its cell-rounding activity and delay virus reproduction in flat cells. J Virol 67:5198-5205.

Barrangou, R., C. Fremaux, H. Deveau, M. Richards, P. Boyaval, S. Moineau, D. A. Romero, and P. Horvath. 2007. CRISPR provides acquired resistance against viruses in prokaryotes. Science 315:1709-1712.

Binsila, K. B., S. Selvaraju, S. K. Ghosh, S. Parthipan, S. S. Archana, A. Arangasamy, J. K. Prasad, R. Bhatta, and J. P. Ravindra. 2018. Isolation and enrichment of putative spermatogonial stem cells from ram (Ovis aries) testis. Anim Reprod Sci 196:9-18.

Boch, J., H. Scholze, S. Schornack, A. Landgraf, S. Hahn, S. Kay, T. Lahaye, A. Nickstadt, and U. Bonas. 2009. Breaking the code of DNA binding specificity of TAL-type III effectors. Science 326:1509-1512.

Boujrad, N., M. T. Hochereau-de Reviers, P. Kamtchouing, C. Perreau, and S. Carreau. 1995. Evolution of somatic and germ cell populations after busulfan treatment in utero or neonatal cryptorchidism in the rat. Andrologia 27:223-228.

Brinster, R. L., and M. R. Avarbock. 1994. Germline transmission of donor haplotype following spermatogonial transplantation. Proc Natl Acad Sci U S A 91:11303-11307.

Brinster, R. L., and J. W. Zimmermann. 1994. Spermatogenesis following male germ-cell transplantation. Proc Natl Acad Sci U S A 91:11298-11302.

Buageaw, A., M. Sukhwani, A. Ben-Yehudah, J. Ehmcke, V. Y. Rawe, C. Pholpramool, K. E. Orwig, and S. Schlatt. 2005. GDNF family receptor alpha1 phenotype of spermatogonial stem cells in immature mouse testes. Biol Reprod 73:1011-1016.

Buo, A. M., M. S. Williams, J. P. Kerr, and J. P. Stains. 2016. A cost-effective method to enhance adenoviral transduction of primary murine osteoblasts and bone marrow stromal cells. Bone Res 4:16021.

Carsience, R. S., M. E. Clark, A. M. Verrinder Gibbins, and R. J. Etches. 1993. Germline chimeric chickens from dispersed donor blastodermal cells and compromised recipient embryos. Development 117:669-675.

Chang, I.-K., M. Naito, T. Kuwana, M. Mizutani, and M. Sakurai. 1998. Production of Germline Chimeric Quail by Transfer of Gonadal Primordial Germ Cells Preserved in Liquid Nitrogen.

Chang, I. K., A. Tajima, Y. Yasuda, T. Chikamune, and T. Ohno. 1992. Simple Method for Isolation of Primordial Germ-Cell from Chick-Embryos. Cell Biol Int Rep 16:853-857.

Chen, Y. C., S. P. Lin, Y. Y. Chang, W. P. Chang, L. Y. Wei, H. C. Liu, J. F. Huang, B. Pain, and S. C. Wu. 2019. In vitro culture and characterization of duck primordial germ cells. Poult Sci 98:1820-1832.

Cheng, R., J. Peng, Y. Yan, P. Cao, J. Wang, C. Qiu, L. Tang, D. Liu, L. Tang, J. Jin, X. Huang, F. He, and P. Zhang. 2014. Efficient gene editing in adult mouse livers via adenoviral delivery of CRISPR/Cas9. FEBS Lett 588:3954-3958.

Chiarini-Garcia, H., and L. D. Russell. 2001. High-resolution light microscopic characterization of mouse spermatogonia. Biol Reprod 65:1170-1178.

Choi, H. J., H. C. Lee, K. S. Kang, H. G. Lee, T. Ono, H. Nagai, G. Sheng, and J. Y. Han. 2015. Production of Interspecific Germline Chimeras via Embryo Replacement. Biol Reprod 93:36.

Choi, J. W., S. Kim, T. M. Kim, Y. M. Kim, H. W. Seo, T. S. Park, J. W. Jeong, G. Song, and J. Y. Han. 2010. Basic fibroblast growth factor activates MEK/ERK cell signaling pathway and stimulates the proliferation of chicken primordial germ cells. Plos One 5:e12968.

Crystal, R. G. 2014. Adenovirus: the first effective in vivo gene delivery vector. Hum Gene Ther 25:3-11.

de Barros, F. R., R. A. Worst, G. C. Saurin, C. M. Mendes, M. E. Assumpcao, and J. A. Visintin. 2012. alpha-6 integrin expression in bovine spermatogonial cells purified by discontinuous Percoll density gradient. Reprod Domest Anim 47:887-890.

de Rooij, D. G. 2017. The nature and dynamics of spermatogonial stem cells. Development 144:3022-3030.

de Rooij, D. G., and L. D. Russell. 2000. All You Wanted to Know About Spermatogonia but Were Afraid to Ask. Journal of Andrology 21:776-798.

Dimitrov, L., D. Pedersen, K. H. Ching, H. Yi, E. J. Collarini, S. Izquierdo, M. C. van de Lavoir, and P. A. Leighton. 2016. Germline Gene Editing in Chickens by Efficient CRISPR-Mediated Homologous Recombination in Primordial Germ Cells. Plos One 11:e0154303.

Douglas, J. T. 2007. Adenoviral vectors for gene therapy. Mol Biotechnol 36:71-80.

Economides, K. D., L. Zeltser, and M. R. Capecchi. 2003. Hoxb13 mutations cause overgrowth of caudal spinal cord and tail vertebrae. Dev Biol 256:317-330.

Ehrke-Schulz, E., M. Schiwon, T. Leitner, S. David, T. Bergmann, J. Liu, and A. Ehrhardt. 2017. CRISPR/Cas9 delivery with one single adenoviral vector devoid of all viral genes. Sci Rep 7:17113.

Eyal-Giladi, H., and S. Kochav. 1976. From cleavage to primitive streak formation: a complementary normal table and a new look at the first stages of the development of the chick. I. General morphology. Dev Biol 49:321-337.

Eyalgiladi, H., M. Ginsburg, and A. Farbarov. 1981. Avian Primordial Germ-Cells Are of Epiblastic Origin. J Embryol Exp Morph 65:139-147.

Fayomi, A. P., and K. E. Orwig. 2018. Spermatogonial stem cells and spermatogenesis in mice, monkeys and men. Stem Cell Res 29:207-214.

Fernandes, L. G. V., L. P. Guaman, S. A. Vasconcellos, M. B. Heinemann, M. Picardeau, and A. Nascimento. 2019. Gene silencing based on RNA-guided catalytically inactive Cas9 (dCas9): a new tool for genetic engineering in Leptospira. Sci Rep 9:1839.

Freimuth, P., K. Springer, C. Berard, J. Hainfeld, M. Bewley, and J. Flanagan. 1999. Coxsackievirus and adenovirus receptor amino-terminal immunoglobulin V-related domain binds adenovirus type 2 and fiber knob from adenovirus type 12. J Virol 73:1392-1398.

Gabriel, R., A. Lombardo, A. Arens, J. C. Miller, P. Genovese, C. Kaeppel, A. Nowrouzi, C. C. Bartholomae, J. Wang, G. Friedman, M. C. Holmes, P. D. Gregory, H. Glimm, M. Schmidt, L. Naldini, and C. von Kalle. 2011. An unbiased genome-wide analysis of zinc-finger nuclease specificity. Nat Biotechnol 29:816-823.

Gao, M., B. Zhang, Y. He, Q. Yang, L. Deng, Y. Zhu, E. Lai, M. Wang, L. Wang, G. Yang, G. Liao, J. Bao, and H. Bu. 2019. Efficient Generation of an Fah/Rag2 Dual-Gene Knockout Porcine Cell Line Using CRISPR/Cas9 and Adenovirus. DNA Cell Biol 38:314-321.

Garbuzov, A., M. F. Pech, K. Hasegawa, M. Sukhwani, R. J. Zhang, K. E. Orwig, and S. E. Artandi. 2018. Purification of GFRalpha1+ and GFRalpha1- Spermatogonial Stem Cells Reveals a Niche-Dependent Mechanism for Fate Determination. Stem Cell Reports 10:553-567.

Gatesy, S. M., and K. P. Dial. 1996. From Frond to Fan: Archaeopteryx and the Evolution of Short-Tailed Birds. Evolution 50:2037-2048.

Gaudelli, N. M., A. C. Komor, H. A. Rees, M. S. Packer, A. H. Badran, D. I. Bryson, and D. R. Liu. 2017. Programmable base editing of A*T to G*C in genomic DNA without DNA cleavage. Nature 551:464-471.

Giansanti, F., L. Leboffe, G. Pitari, R. Ippoliti, and G. Antonini. 2012. Physiological roles of ovotransferrin. Biochim Biophys Acta 1820:218-225.

Giassetti, M. I., M. D. Goissis, F. de Barros, A. H. Bruno, M. Assumpcao, and J. A. Visintin. 2016. Comparison of Diverse Differential Plating Methods to Enrich Bovine Spermatogonial Cells. Reprod Domest Anim 51:26-32.

Ginsburg, M., and H. Eyalgiladi. 1987. Primordial Germ-Cells of the Young Chick Blastoderm Originate from the Central Zone of the Area Pellucida Irrespective of the Embryo-Forming Process. Development 101:209-219.

Ginsburg, M., and H. Eyalgiladi. 1989. Primordial Germ-Cell Development in Cultures of Dispersed Central Disks of Stage-X Chick Blastoderms. Gamete Res 23:421-427.

Goodyear, S., and R. Brinster. 2017. Isolation of the Spermatogonial Stem Cell-Containing Fraction from Testes. Cold Spring Harb Protoc 2017:pdb prot094185.

Goossens, E., D. Van Saen, and H. Tournaye. 2013. Spermatogonial stem cell preservation and transplantation: from research to clinic. Hum Reprod 28:897-907.

Gwiazda, K. S., A. E. Grier, J. Sahni, S. M. Burleigh, U. Martin, J. G. Yang, N. A. Popp, M. C. Krutein, I. F. Khan, K. Jacoby, M. C. Jensen, D. J. Rawlings, and A. M. Scharenberg. 2016. High Efficiency CRISPR/Cas9-mediated Gene Editing in Primary Human T-cells Using Mutant Adenoviral E4orf6/E1b55k "Helper" Proteins. Mol Ther 24:1570-1580.

Hall, S. J., N. Bar-Chama, S. Ta, and J. W. Gordon. 2000. Direct exposure of mouse spermatogenic cells to high doses of adenovirus gene therapy vector does not result in germ cell transduction. Hum Gene Ther 11:1705-1712.

Hamburger, V., and H. L. Hamilton. 1951. A Series of Normal Stages in the Development of the Chick Embryo. J Morphol 88:49-&.

Hamra, F. K., J. Gatlin, K. M. Chapman, D. M. Grellhesl, J. V. Garcia, R. E. Hammer, and D. L. Garbers. 2002. Production of transgenic rats by lentiviral transduction of male germ-line stem cells. Proc Natl Acad Sci U S A 99:14931-14936.

Han, J., H. Lee, and T. Park. 2015. Germline-competent stem cell in avian species and its application. Asian Journal of Andrology 17:421-426.

Han, J. Y., H. G. Lee, Y. S. Hwang, H. C. Lee, S. K. Kim, and D. Rengaraj. 2018. Expression of transcription factors during area pellucida formation in intrauterine chicken embryos. Int J Dev Biol 62:341-345.

Han, J. Y., T. S. Park, Y. H. Hong, D. K. Jeong, J. N. Kim, K. D. Kim, and J. M. Lim. 2002. Production of germline chimeras by transfer of chicken gonadal primordial germ cells maintained in vitro for an extended period. Theriogenology 58:1531-1539.

Han, J. Y., and Y. H. Park. 2018. Primordial germ cell-mediated transgenesis and genome editing in birds. Journal of animal science and biotechnology 9:19-19.

Herrid, M., J. Olejnik, M. Jackson, N. Suchowerska, S. Stockwell, R. Davey, K. Hutton, S. Hope, and J. R. Hill. 2009. Irradiation Enhances the Efficiency of Testicular Germ Cell Transplantation in Sheep. Biol Reprod 81:898-905.

Herrid, M., S. Vignarajan, R. Davey, I. Dobrinski, and J. R. Hill. 2006. Successful transplantation of bovine testicular cells to heterologous recipients. Reproduction 132:617-624.

Herron, L. R., C. Pridans, M. L. Turnbull, N. Smith, S. Lillico, A. Sherman, H. J. Gilhooley, M. Wear, D. Kurian, G. Papadakos, P. Digard, D. A. Hume, A. C. Gill, and H. M. Sang. 2018. A chicken bioreactor for efficient production of functional cytokines. BMC Biotechnol 18:82.

Honaramooz, A., E. Behboodi, S. O. Megee, S. A. Overton, H. Galantino-Homer, Y. Echelard, and I. Dobrinski. 2003. Fertility and germline transmission of donor haplotype following germ cell transplantation in immunocompetent goats. Biol Reprod 69:1260-1264.

Honaramooz, A., S. Megee, W. Zeng, M. M. Destrempes, S. A. Overton, J. Luo, H. Galantino-Homer, M. Modelski, F. Chen, S. Blash, D. T. Melican, W. G. Gavin, S. Ayres, F. Yang, P. J. Wang, Y. Echelard, and I. Dobrinski. 2008. Adeno-associated virus (AAV)-mediated transduction of male germ line stem cells results in transgene transmission after germ cell transplantation. Faseb J 22:374-382.

Honda, T., H. Saitoh, M. Masuko, T. Katagiri-Abe, K. Tominaga, I. Kozakai, K. Kobayashi, T. Kumanishi, Y. G. Watanabe, S. Odani, and R. Kuwano. 2000. The coxsackievirus-adenovirus receptor protein as a cell adhesion molecule in the developing mouse brain. Brain Res Mol Brain Res 77:19-28.

Hu, J. H., S. M. Miller, M. H. Geurts, W. Tang, L. Chen, N. Sun, C. M. Zeina, X. Gao, H. A. Rees, Z. Lin, and D. R. Liu. 2018. Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. Nature 556:57-63.

Hur, J. K., K. Kim, K. W. Been, G. Baek, S. Ye, J. W. Hur, S. M. Ryu, Y. S. Lee, and J. S. Kim. 2016. Targeted mutagenesis in mice by electroporation of Cpf1 ribonucleoproteins. Nat Biotechnol 34:807-808.

Huss, D., B. Benazeraf, A. Wallingford, M. Filla, J. Yang, S. E. Fraser, and R. Lansford. 2015. A transgenic quail model that enables dynamic imaging of amniote embryogenesis. Development 142:2850-2859.

Ivarie, R. 2003. Avian transgenesis: progress towards the promise. Trends Biotechnol 21:14-19.

Izadyar, F., K. Den Ouden, T. A. Stout, J. Stout, J. Coret, D. P. Lankveld, T. J. Spoormakers, B. Colenbrander, J. K. Oldenbroek, K. D. Van der Ploeg, H. Woelders, H. B. Kal, and D. G. De Rooij. 2003. Autologous and homologous transplantation of bovine spermatogonial stem cells. Reproduction 126:765-774.

Izadyar, F., G. T. Spierenberg, L. B. Creemers, K. den Ouden, and D. G. de Rooij. 2002. Isolation and purification of type A spermatogonia from the bovine testis. Reproduction 124:85-94.

Izsvak, Z., J. Frohlich, I. Grabundzija, J. R. Shirley, H. M. Powell, K. M. Chapman, Z. Ivics, and F. K. Hamra. 2010. Generating knockout rats by transposon mutagenesis in spermatogonial stem cells. Nat Methods 7:443-445.

Jegou, B. 1993. The Sertoli-germ cell communication network in mammals. Int Rev Cytol 147:25-96.

Jin, Y. H., H. Joo, K. Lee, H. Kim, R. Didier, Y. Yang, H. Shin, and C. Lee. 2019. Streamlined procedure for gene knockouts using all-in-one adenoviral CRISPR-Cas9. Sci Rep 9:277.

Jinek, M., K. Chylinski, I. Fonfara, M. Hauer, J. A. Doudna, and E. Charpentier. 2012. A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity. Science 337:816-821.

Jones, P., E. Kominkova, and H. Jackson. 1972. Effects of antifertility substances on male Japanese quail. J Reprod Fertil 29:71-78.

Jung, J. G., Y. M. Lee, T. S. Park, S. H. Park, J. M. Lim, and J. Y. Han. 2007. Identification, culture, and characterization of germline stem cell-like cells in chicken testes. Biol Reprod 76:173-182.

Jung, K. M., Y. M. Kim, T. Ono, and J. Y. Han. 2017. Size-dependent isolation of primordial germ cells from avian species. Mol Reprod Dev 84:508-516.

Kanatsu-Shinohara, M., M. Kato-Itoh, M. Ikawa, M. Takehashi, M. Sanbo, Y. Morioka, T. Tanaka, H. Morimoto, M. Hirabayashi, and T. Shinohara. 2011. Homologous recombination in rat germline stem cells. Biol Reprod 85:208-217.

Kanatsu-Shinohara, M., H. Morimoto, and T. Shinohara. 2012. Enrichment of mouse spermatogonial stem cells by melanoma cell adhesion molecule expression. Biol Reprod 87:139.

Kanatsu-Shinohara, M., H. Morimoto, and T. Shinohara. 2016. Fertility of Male Germline Stem Cells Following Spermatogonial Transplantation in Infertile Mouse Models. Biol Reprod 94:112.

Kanatsu-Shinohara, M., A. Ogura, M. Ikegawa, K. Inoue, N. Ogonuki, K. Tashiro, S. Toyokuni, T. Honjo, and T. Shinohara. 2002. Adenovirus-mediated gene delivery and in vitro microinsemination produce offspring from infertile male mice. Proc Natl Acad Sci U S A 99:1383-1388.

Kanatsu-Shinohara, M., S. Toyokuni, and T. Shinohara. 2004. CD9 is a surface marker on mouse and rat male germline stem cells. Biol Reprod 70:70-75.

Kang, S. J., J. W. Choi, S. Y. Kim, K. J. Park, T. M. Kim, Y. M. Lee, H. Kim, J. M. Lim, and J. Y. Han. 2008. Reproduction of wild birds via interspecies germ cell transplantation. Biol Reprod 79:931-937.

Kasman, L. M., S. Barua, P. Lu, K. Rege, and C. Voelkel-Johnson. 2009. Polymer-enhanced adenoviral transduction of CAR-negative bladder cancer cells. Mol Pharm 6:1612-1619.

Kim, E., T. Koo, S. W. Park, D. Kim, K. Kim, H. Y. Cho, D. W. Song, K. J. Lee, M. H. Jung, S. Kim, J. H. Kim, J. H. Kim, and J. S. Kim. 2017a. In vivo genome editing with a small Cas9 orthologue derived from Campylobacter jejuni. Nat Commun 8:14500.

Kim, H., and J. S. Kim. 2014. A guide to genome engineering with programmable nucleases. Nat Rev Genet 15:321-334.

Kim, J. H., N. Sharma, S. W. Kim, S. S. Sodhi, M. Ghosh, N. E. Kim, R. K. Mongre, S. J. Oh, and D. K. Jeong. 2014. Establishment of a pheasant (Phasianus colchicus) spermatogonial stem cell line for the production of interspecies germ line chimeras. Electronic Journal of Biotechnology 17:211-216.

Kim, M. A., T. S. Park, J. N. Kim, H. J. Park, Y. M. Lee, T. Ono, J. M. Lim, and J. Y. Han. 2005. Production of quail (Coturnix japonica) germline chimeras by transfer of gonadal primordial germ cells into recipient embryos. Theriogenology 63:774-782.

Kim, S. W., J. H. Lee, B. C. Park, and T. S. Park. 2017b. Myotube differentiation in clustered regularly interspaced short palindromic repeat/Cas9-mediated MyoD knockout quail myoblast cells. Asian-Australas J Anim Sci 30:1029-1036.

Kim, Y., D. Turner, J. Nelson, I. Dobrinski, M. McEntee, and A. J. Travis. 2008. Production of donor-derived sperm after spermatogonial stem cell transplantation in the dog. Reproduction 136:823-831.

Kim, Y. G., J. Cha, and S. Chandrasegaran. 1996. Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. Proc Natl Acad Sci U S A 93:1156-1160.

Kim, Y. M. 2018. Studies on avian germline competent stem cells and practical application for bioreactor system. 서울대학교 대학원.

Kim, Y. M., J. S. Park, J. W. Yoon, H. J. Choi, K. J. Park, T. Ono, and J. Y. Han. 2018. Production of germline chimeric quails following spermatogonial cell transplantation in busulfan-treated testis. Asian J Androl 20:414-416.

Kokkinaki, M., A. Djourabtchi, and N. Golestaneh. 2011. Long-term Culture of Human SSEA-4 Positive Spermatogonial Stem Cells (SSCs). J Stem Cell Res Ther 2.

Kokkinaki, M., T. L. Lee, Z. He, J. Jiang, N. Golestaneh, M. C. Hofmann, W. Y. Chan, and M. Dym. 2009. The molecular signature of spermatogonial stem/progenitor cells in the 6-day-old mouse testis. Biol Reprod 80:707-717.

Koruji, M., M. Movahedin, S. J. Mowla, H. Gourabi, S. Pour-Beiranvand, and A. Jabbari Arfaee. 2012. Autologous transplantation of adult mice spermatogonial stem cells into gamma irradiated testes. Cell J 14:82-89.

Kossack, N., J. Meneses, S. Shefi, H. N. Nguyen, S. Chavez, C. Nicholas, J. Gromoll, P. J. Turek, and R. A. Reijo-Pera. 2009. Isolation and characterization of pluripotent human spermatogonial stem cell-derived cells. Stem Cells 27:138-149.

Kuijk, E. W., B. Colenbrander, and B. A. Roelen. 2009. The effects of growth factors on in vitro-cultured porcine testicular cells. Reproduction 138:721-731.

Kwon, S. C., J. W. Choi, H. J. Jang, S. S. Shin, S. K. Lee, T. S. Park, I. Y. Choi, G. S. Lee, G. Song, and J. Y. Han. 2010. Production of biofunctional recombinant human interleukin 1 receptor antagonist (rhIL1RN) from transgenic quail egg white. Biol Reprod 82:1057-1064.

Lacerda, S. M., G. M. Costa, and L. R. de Franca. 2014. Biology and identity of fish spermatogonial stem cell. Gen Comp Endocrinol 207:56-65.

Lanza, R. P., J. B. Cibelli, F. Diaz, C. T. Moraes, P. W. Farin, C. E. Farin, C. J. Hammer, M. D. West, and P. Damiani. 2000. Cloning of an endangered species (Bos gaurus) using interspecies nuclear transfer. Cloning 2:79-90.

Lasaro, M. O., and H. C. Ertl. 2009. New insights on adenovirus as vaccine vectors. Mol Ther 17:1333-1339.

Lau, C. H., and Y. Suh. 2017. In vivo genome editing in animals using AAV-CRISPR system: applications to translational research of human disease. F1000Res 6:2153.

Lee, C. S., E. S. Bishop, R. Zhang, X. Yu, E. M. Farina, S. Yan, C. Zhao, Z. Zheng, Y. Shu, X. Wu, J. Lei, Y. Li, W. Zhang, C. Yang, K. Wu, Y. Wu, S. Ho, A. Athiviraham, M. J. Lee, J. M. Wolf, R. R. Reid, and T. C. He. 2017. Adenovirus-Mediated Gene Delivery: Potential Applications for Gene and Cell-Based Therapies in the New Era of Personalized Medicine. Genes Dis 4:43-63.

Lee, H. C., H. J. Choi, T. S. Park, S. I. Lee, Y. M. Kim, D. Rengaraj, H. Nagai, G. Sheng, J. M. Lim, and J. Y. Han. 2013. Cleavage events and sperm dynamics in chick intrauterine embryos. Plos One 8:e80631.

Lee, H. J., H. C. Lee, Y. M. Kim, Y. S. Hwang, Y. H. Park, T. S. Park, and J. Y. Han. 2016. Site-specific recombination in the chicken genome using Flipase recombinase–mediated cassette exchange. The FASEB Journal 30:555-563.

Lee, J., J. Ma, and K. Lee. 2019. Direct delivery of adenoviral CRISPR/Cas9 vector into the blastoderm for generation of targeted gene knockout in quail. Proc Natl Acad Sci U S A.

Lee, Y. M., J. G. Jung, J. N. Kim, T. S. Park, T. M. Kim, S. S. Shin, D. K. Kang, J. M. Lim, and J. Y. Han. 2006a. A testis-mediated germline chimera production based on transfer of chicken testicular cells directly into heterologous testes. Biol Reprod 75:380-386.

Lee, Y. M., J. G. Jung, J. N. Kim, T. S. Park, T. M. Kim, S. S. Shin, D. K. Kang, J. M. Lim, and J. Y. Han. 2006b. A testis-mediated germline chimera production based on transfer of chicken testicular cells directly into heterologous testes. Biol Reprod 75:380-386.

Li, C., N. Psatha, S. Gil, H. Wang, T. Papayannopoulou, and A. Lieber. 2018. HDAd5/35(++) Adenovirus Vector Expressing Anti-CRISPR Peptides Decreases CRISPR/Cas9 Toxicity in Human Hematopoietic Stem Cells. Mol Ther Methods Clin Dev 9:390-401.

Li, C. H., L. Z. Yan, W. Z. Ban, Q. Tu, Y. Wu, L. Wang, R. Bi, S. Ji, Y. H. Ma, W. H. Nie, L. B. Lv, Y. G. Yao, X. D. Zhao, and P. Zheng. 2017. Long-term propagation of tree shrew spermatogonial stem cells in culture and successful generation of transgenic offspring. Cell Res 27:241-252.

Li, E., D. Stupack, G. M. Bokoch, and G. R. Nemerow. 1998a. Adenovirus endocytosis requires actin cytoskeleton reorganization mediated by Rho family GTPases. J Virol 72:8806-8812.

Li, E., D. Stupack, R. Klemke, D. A. Cheresh, and G. R. Nemerow. 1998b. Adenovirus endocytosis via alpha(v) integrins requires phosphoinositide-3-OH kinase. J Virol 72:2055-2061.

Li, L., A. Atef, A. Piatek, Z. Ali, M. Piatek, M. Aouida, A. Sharakuu, A. Mahjoub, G. Wang, S. Khan, N. V. Fedoroff, J. K. Zhu, and M. M. Mahfouz. 2013. Characterization and DNA-binding specificities of Ralstonia TAL-like effectors. Mol Plant 6:1318-1330.

Li, X., and W. D. Heyer. 2008. Homologous recombination in DNA repair and DNA damage tolerance. Cell Res 18:99-113.

Liao, H. K., F. Hatanaka, T. Araoka, P. Reddy, M. Z. Wu, Y. Sui, T. Yamauchi, M. Sakurai, D. D. O'Keefe, E. Nunez-Delicado, P. Guillen, J. M. Campistol, C. J. Wu, L. F. Lu, C. R. Esteban, and J. C. Izpisua Belmonte. 2017. In Vivo Target Gene Activation via CRISPR/Cas9-Mediated Trans-epigenetic Modulation. Cell 171:1495-1507 e1415.

Lim, J. J., H. J. Kim, K. S. Kim, J. Y. Hong, and D. R. Lee. 2013. In vitro culture-induced pluripotency of human spermatogonial stem cells. Biomed Res Int 2013:143028.

Lin, Z., J. Bao, Q. Kong, Y. Bai, F. Luo, Z. Songyang, Y. Wu, and J. Huang. 2017. Effective production of recipient male pigs for spermatogonial stem cell transplantation by intratesticular injection with busulfan. Theriogenology 89:365-373 e362.

Liu, C. H., K. A. Khazanehdari, V. Baskar, S. Saleem, J. Kinne, U. Wernery, and I. K. Chang. 2012. Production of Chicken Progeny (Gallus gallus domesticus) from Interspecies Germline Chimeric Duck (Anas domesticus) by Primordial Germ Cell Transfer. Biol Reprod 86.

Liu, S., Z. Tang, T. Xiong, and W. Tang. 2011. Isolation and characterization of human spermatogonial stem cells. Reprod Biol Endocrinol 9:141.

Loi, P., G. Ptak, B. Barboni, J. Fulka, Jr., P. Cappai, and M. Clinton. 2001. Genetic rescue of an endangered mammal by cross-species nuclear transfer using post-mortem somatic cells. Nat Biotechnol 19:962-964.

Lundstrom, K. 2018. Viral Vectors in Gene Therapy. Diseases 6.

Macdonald, J., J. D. Glover, L. Taylor, H. M. Sang, and M. J. McGrew. 2010. Characterisation and germline transmission of cultured avian primordial germ cells. Plos One 5:e15518.

Macdonald, J., L. Taylor, A. Sherman, K. Kawakami, Y. Takahashi, H. M. Sang, and M. J. McGrew. 2012. Efficient genetic modification and germ-line transmission of primordial germ cells using piggyBac and Tol2 transposons. Proc Natl Acad Sci U S A 109:E1466-1472.

Maezawa, S., K. Hasegawa, K. G. Alavattam, M. Funakoshi, T. Sato, A. Barski, and S. H. Namekawa. 2018. SCML2 promotes heterochromatin organization in late spermatogenesis. J Cell Sci 131.

Maggio, I., M. Holkers, J. Liu, J. M. Janssen, X. Chen, and M. A. Goncalves. 2014. Adenoviral vector delivery of RNA-guided CRISPR/Cas9 nuclease complexes induces targeted mutagenesis in a diverse array of human cells. Sci Rep 4:5105.

Meng, X., M. Lindahl, M. E. Hyvonen, M. Parvinen, D. G. de Rooij, M. W. Hess, A. Raatikainen-Ahokas, K. Sainio, H. Rauvala, M. Lakso, J. G. Pichel, H. Westphal, M. Saarma, and H. Sariola. 2000. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science 287:1489-1493.

Meyer, D. B. 1964. The Migration of Primordial Germ Cells in the Chick Embryo. Dev Biol 10:154-190.

Miller, J. C., S. Tan, G. Qiao, K. A. Barlow, J. Wang, D. F. Xia, X. Meng, D. E. Paschon, E. Leung, S. J. Hinkley, G. P. Dulay, K. L. Hua, I. Ankoudinova, G. J. Cost, F. D. Urnov, H. S. Zhang, M. C. Holmes, L. Zhang, P. D. Gregory, and E. J. Rebar. 2011. A TALE nuclease architecture for efficient genome editing. Nat Biotechnol 29:143-148.

Mojica, F. J., C. Diez-Villasenor, J. Garcia-Martinez, and C. Almendros. 2009. Short motif sequences determine the targets of the prokaryotic CRISPR defence system. Microbiology 155:733-740.

Momeni-Moghaddam, M., M. M. Matin, S. Boozarpour, S. Sisakhtnezhad, H. K. Mehrjerdi, M. Farshchian, M. Dastpak, and A. R. Bahrami. 2014. A simple method for isolation, culture, and in vitro maintenance of chicken spermatogonial stem cells. In Vitro Cell Dev Biol Anim 50:155-161.

Motono, M., Y. Yamada, Y. Hattori, R. Nakagawa, K. Nishijima, and S. Iijima. 2010. Production of transgenic chickens from purified primordial germ cells infected with a lentiviral vector. J Biosci Bioeng 109:315-321.

Moulavi, F., S. M. Hosseini, N. Tanhaie-Vash, S. Ostadhosseini, S. H. Hosseini, M. Hajinasrollah, M. H. Asghari, H. Gourabi, A. Shahverdi, A. D. Vosough, and M. H. Nasr-Esfahani. 2017. Interspecies somatic cell nuclear transfer in Asiatic cheetah using nuclei derived from post-mortem frozen tissue in absence of cryo-protectant and in vitro matured domestic cat oocytes. Theriogenology 90:197-203.

Mozdziak, P. E., J. Angerman-Stewart, B. Rushton, S. L. Pardue, and J. N. Petitte. 2005. Isolation of chicken primordial germ cells using fluorescence-activated cell sorting. Poultry Sci 84:594-600.

Mussolino, C., and T. Cathomen. 2012. TALE nucleases: tailored genome engineering made easy. Curr Opin Biotechnol 23:644-650.

Nagano, M., B. Y. Ryu, C. J. Brinster, M. R. Avarbock, and R. L. Brinster. 2003. Maintenance of mouse male germ line stem cells in vitro. Biol Reprod 68:2207-2214.

Naito, M., T. Harumi, and T. Kuwana. 2015. Long-term culture of chicken primordial germ cells isolated from embryonic blood and production of germline chimaeric chickens. Animal Reproduction Science 153:50-61.

Naito, M., A. Tajima, Y. Yasuda, and T. Kuwana. 1994. Production of Germline Chimeric Chickens, with High Transmission Rate of Donor-Derived Gametes, Produced by Transfer of Primordial Germ-Cells. Mol Reprod Dev 39:153-161.

Nakamura, Y., M. Tasai, K. Takeda, K. Nirasawa, and T. Tagami. 2013. Production of functional gametes from cryopreserved primordial germ cells of the Japanese quail. J Reprod Dev 59:580-587.

Nasiri, Z., S. M. Hosseini, M. Hajian, P. Abedi, M. Bahadorani, H. Baharvand, and M. H. Nasr-Esfahani. 2012. Effects of different feeder layers on short-term culture of prepubertal bovine testicular germ cells in-vitro. Theriogenology 77:1519-1528.

Oakberg, E. F. 1971. Spermatogonial stem-cell renewal in the mouse. Anat Rec 169:515-531.

Ogawa, T., I. Dobrinski, and R. L. Brinster. 1999. Recipient preparation is critical for spermatogonial transplantation in the rat. Tissue Cell 31:461-472.

Ohmura, M., S. Yoshida, Y. Ide, G. Nagamatsu, T. Suda, and K. Ohbo. 2004. Spatial analysis of germ stem cell development in Oct-4/EGFP transgenic mice. Arch Histol Cytol 67:285-296.

Oishi, I., K. Yoshii, D. Miyahara, H. Kagami, and T. Tagami. 2016. Targeted mutagenesis in chicken using CRISPR/Cas9 system. Sci Rep 6:23980.

Ono, T., and Y. Machida. 1999. Immunomagnetic purification of viable primordial germ cells of Japanese quail (Coturnix japonica). Comp Biochem Phys A 122:255-259.

Ono, T., T. Matsumoto, and Y. Arisawa. 1998. Production of donor-derived offspring by transfer of primordial germ cells in Japanese quail. Exp Anim 47:215-219.

Pain, B., M. E. Clark, M. Shen, H. Nakazawa, M. Sakurai, J. Samarut, and R. J. Etches. 1996. Long-term in vitro culture and characterisation of avian embryonic stem cells with multiple morphogenetic potentialities. Development 122:2339-2348.

Panda, R. P., H. K. Barman, and C. Mohapatra. 2011. Isolation of enriched carp spermatogonial stem cells from Labeo rohita testis for in vitro propagation. Theriogenology 76:241-251.

Park, T. S., and J. Y. Han. 2012. piggyBac transposition into primordial germ cells is an efficient tool for transgenesis in chickens. Proc Natl Acad Sci U S A 109:9337-9341.

Park, T. S., M. A. Kim, J. M. Lim, and J. Y. Han. 2008. Production of quail (Coturnix japonica) germline chimeras derived from in vitro-cultured gonadal primordial germ cells. Mol Reprod Dev 75:274-281.

Park, T. S., H. J. Lee, K. H. Kim, J. S. Kim, and J. Y. Han. 2014. Targeted gene knockout in chickens mediated by TALENs. Proc Natl Acad Sci U S A 111:12716-12721.

Parks, R. J., L. Chen, M. Anton, U. Sankar, M. A. Rudnicki, and F. L. Graham. 1996. A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. Proc Natl Acad Sci U S A 93:13565-13570.

Pattanayak, V., C. L. Ramirez, J. K. Joung, and D. R. Liu. 2011. Revealing off-target cleavage specificities of zinc-finger nucleases by in vitro selection. Nat Methods 8:765-770.

Peters, A. H., J. Drumm, C. Ferrell, D. A. Roth, D. M. Roth, M. McCaman, P. L. Novak, J. Friedman, R. Engler, and R. E. Braun. 2001. Absence of germline infection in male mice following intraventricular injection of adenovirus. Mol Ther 4:603-613.

Petitte, J. N., M. E. Clark, G. Liu, A. M. Verrinder Gibbins, and R. J. Etches. 1990. Production of somatic and germline chimeras in the chicken by transfer of early blastodermal cells. Development 108:185-189.

Phillips, B. T., K. Gassei, and K. E. Orwig. 2010. Spermatogonial stem cell regulation and spermatogenesis. Philosophical Transactions of the Royal Society B: Biological Sciences 365:1663-1678.

Poynter, G., D. Huss, and R. Lansford. 2009. Injection of lentivirus into stage-X blastoderm for the production of transgenic quail. Cold Spring Harb Protoc 2009:pdb prot5118.

Pramod, R. K., B. R. Lee, Y. M. Kim, H. J. Lee, Y. H. Park, T. Ono, J. M. Lim, and J. Y. Han. 2017. Isolation, Characterization, and In Vitro Culturing of Spermatogonial Stem Cells in Japanese Quail (Coturnix japonica). Stem Cells Dev 26:60-70.

Ran, F. A., L. Cong, W. X. Yan, D. A. Scott, J. S. Gootenberg, A. J. Kriz, B. Zetsche, O. Shalem, X. Wu, K. S. Makarova, E. V. Koonin, P. A. Sharp, and F. Zhang. 2015. In vivo genome editing using Staphylococcus aureus Cas9. Nature 520:186-191.

Rashid, D. J., S. C. Chapman, H. C. Larsson, C. L. Organ, A. G. Bebin, C. S. Merzdorf, R. Bradley, and J. R. Horner. 2014. From dinosaurs to birds: a tail of evolution. Evodevo 5:25.

Rauschhuber, C., N. Noske, and A. Ehrhardt. 2012. New insights into stability of recombinant adenovirus vector genomes in mammalian cells. Eur J Cell Biol 91:2-9.

Regan, E. C., L. Santini, L. Ingwall-King, M. Hoffmann, C. Rondinini, A. Symes, J. Taylor, and S. H. M. Butchart. 2015. Global Trends in the Status of Bird and Mammal Pollinators. Conservation Letters 8:397-403.

Reynaud, G. 1969. [The transfer of turkey primordial germ cells to chick embryos by intravascular injection]. J Embryol Exp Morphol 21:485-507.

Rodriguez-Sosa, J. R., H. Dobson, and A. Hahnel. 2006. Isolation and transplantation of spermatogonia in sheep. Theriogenology 66:2091-2103.

Roe, M., N. McDonald, B. Durrant, and T. Jensen. 2013. Xenogeneic transfer of adult quail (Coturnix coturnix) spermatogonial stem cells to embryonic chicken (Gallus gallus) hosts: a model for avian conservation. Biol Reprod 88:129.

Rowe, W. P., R. J. Huebner, L. K. Gilmore, R. H. Parrott, and T. G. Ward. 1953. Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. Proc Soc Exp Biol Med 84:570-573.

Ryu, B. Y., K. E. Orwig, H. Kubota, M. R. Avarbock, and R. L. Brinster. 2004. Phenotypic and functional characteristics of spermatogonial stem cells in rats. Dev Biol 274:158-170.

Salter, D. W., E. J. Smith, S. H. Hughes, S. E. Wright, A. M. Fadly, R. L. Witter, and L. B. Crittenden. 1986. Gene insertion into the chicken germ line by retroviruses. Poult Sci 65:1445-1458.

Sato, T., T. Sakuma, T. Yokonishi, K. Katagiri, S. Kamimura, N. Ogonuki, A. Ogura, T. Yamamoto, and T. Ogawa. 2015. Genome Editing in Mouse Spermatogonial Stem Cell Lines Using TALEN and Double-Nicking CRISPR/Cas9. Stem Cell Reports 5:75-82.

Sato, Y., G. Poynter, D. Huss, M. B. Filla, A. Czirok, B. J. Rongish, C. D. Little, S. E. Fraser, and R. Lansford. 2010. Dynamic analysis of vascular morphogenesis using transgenic quail embryos. Plos One 5:e12674.

Satomura, A., R. Nishioka, H. Mori, K. Sato, K. Kuroda, and M. Ueda. 2017. Precise genome-wide base editing by the CRISPR Nickase system in yeast. Sci Rep 7:2095.

Schiwon, M., E. Ehrke-Schulz, A. Oswald, T. Bergmann, T. Michler, U. Protzer, and A. Ehrhardt. 2018. One-Vector System for Multiplexed CRISPR/Cas9 against Hepatitis B Virus cccDNA Utilizing High-Capacity Adenoviral Vectors. Mol Ther Nucleic Acids 12:242-253.

Scott, B. B., and C. Lois. 2005. Generation of tissue-specific transgenic birds with lentiviral vectors. Proc Natl Acad Sci U S A 102:16443-16447.

Seidl, A. H., J. T. Sanchez, L. Schecterson, K. M. Tabor, Y. Wang, D. T. Kashima, G. Poynter, D. Huss, S. E. Fraser, R. Lansford, and E. W. Rubel. 2013. Transgenic quail as a model for research in the avian nervous system: a comparative study of the auditory brainstem. J Comp Neurol 521:5-23.

Selleck, M. A. 1996. Culture and microsurgical manipulation of the early avian embryo. Methods Cell Biol 51:1-21.

Shin, S. S., T. M. Kim, S. Y. Kim, T. W. Kim, H. W. Seo, S. K. Lee, S. C. Kwon, G. S. Lee, H. Kim, J. M. Lim, and J. Y. Han. 2008. Generation of transgenic quail through germ cell-mediated germline transmission. FASEB J 22:2435-2444.

Shinohara, T., M. R. Avarbock, and R. L. Brinster. 1999. beta1- and alpha6-integrin are surface markers on mouse spermatogonial stem cells. Proc Natl Acad Sci U S A 96:5504-5509.

Shinohara, T., and R. L. Brinster. 2000. Enrichment and transplantation of spermatogonial stem cells. Int J Androl 23 Suppl 2:89-91.

Shinohara, T., M. Kato, M. Takehashi, J. Lee, S. Chuma, N. Nakatsuji, M. Kanatsu-Shinohara, and M. Hirabayashi. 2006. Rats produced by interspecies spermatogonial transplantation in mice and in vitro microinsemination. Proc Natl Acad Sci U S A 103:13624-13628.

Shinohara, T., K. E. Orwig, M. R. Avarbock, and R. L. Brinster. 2000. Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. Proc Natl Acad Sci U S A 97:8346-8351.

Smith, J., M. Bibikova, F. G. Whitby, A. R. Reddy, S. Chandrasegaran, and D. Carroll. 2000. Requirements for double-strand cleavage by chimeric restriction enzymes with zinc finger DNA-recognition domains. Nucleic Acids Res 28:3361-3369.

Soh, T., Y. Inoue, Y. M. Xi, Y. Kato, and M. A. Hattori. 2004. Production of interspecific germline chimera between quail and chicken utilizing the cells from central disk of blastoderm and germinal crescent region. Journal of the Faculty of Agriculture, Kyushu University 49:69-75.

Sun, Y., K. Tao, J. J. Tu, W. B. Zhu, and L. Q. Fan. 2008. [Establishment of a long-term culture system for mouse spermatogonial stem cells in vitro]. Zhonghua Nan Ke Xue 14:695-700.

Tagirov, M., and S. Golovan. 2012. The effect of busulfan treatment on endogenous spermatogonial stem cells in immature roosters. Poult Sci 91:1680-1685.

Tajima, A., M. Naito, Y. Yasuda, and T. Kuwana. 1993. Production of Germ-Line Chimera by Transfer of Primordial Germ-Cells in the Domestic Chicken (Gallus-Domesticus). Theriogenology 40:509-519.

Takashima, S., and T. Shinohara. 2018. Culture and transplantation of spermatogonial stem cells. Stem Cell Research 29:46-55.

Takehashi, M., M. Kanatsu-Shinohara, K. Inoue, N. Ogonuki, H. Miki, S. Toyokuni, A. Ogura, and T. Shinohara. 2007. Adenovirus-mediated gene delivery into mouse spermatogonial stem cells. Proceedings of the National Academy of Sciences 104:2596-2601.

Tegelenbosch, R. A., and D. G. de Rooij. 1993. A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. Mutat Res 290:193-200.

Tittensor, D. P., M. Walpole, S. L. Hill, D. G. Boyce, G. L. Britten, N. D. Burgess, S. H. Butchart, P. W. Leadley, E. C. Regan, R. Alkemade, R. Baumung, C. Bellard, L. Bouwman, N. J. Bowles-Newark, A. M. Chenery, W. W. Cheung, V. Christensen, H. D. Cooper, A. R. Crowther, M. J. Dixon, A. Galli, V. Gaveau, R. D. Gregory, N. L. Gutierrez, T. L. Hirsch, R. Hoft, S. R. Januchowski-Hartley, M. Karmann, C. B. Krug, F. J. Leverington, J. Loh, R. K. Lojenga, K. Malsch, A. Marques, D. H. Morgan, P. J. Mumby, T. Newbold, K. Noonan-Mooney, S. N. Pagad, B. C. Parks, H. M. Pereira, T. Robertson, C. Rondinini, L. Santini, J. P. Scharlemann, S. Schindler, U. R. Sumaila, L. S. Teh, J. van Kolck, P. Visconti, and Y. Ye. 2014. A mid-term analysis of progress toward international biodiversity targets. Science 346:241-244.

Trefil, P., A. Micakova, J. Mucksova, J. Hejnar, M. Poplstein, M. R. Bakst, J. Kalina, and J. P. Brillard. 2006. Restoration of spermatogenesis and male fertility by transplantation of dispersed testicular cells in the chicken. Biol Reprod 75:575-581.

Tsukamoto, T., E. Sakai, S. Iizuka, M. Taracena-Gandara, F. Sakurai, and H. Mizuguchi. 2018. Generation of the Adenovirus Vector-Mediated CRISPR/Cpf1 System and the Application for Primary Human Hepatocytes Prepared from Humanized Mice with Chimeric Liver. Biol Pharm Bull 41:1089-1095.

Urnov, F. D., E. J. Rebar, M. C. Holmes, H. S. Zhang, and P. D. Gregory. 2010. Genome editing with engineered zinc finger nucleases. Nat Rev Genet 11:636-646.

van de Lavoir, M. C., E. J. Collarini, P. A. Leighton, J. Fesler, D. R. Lu, W. D. Harriman, T. S. Thiyagasundaram, and R. J. Etches. 2012. Interspecific germline transmission of cultured primordial germ cells. Plos One 7:e35664.

Vannucci, L., M. Lai, F. Chiuppesi, L. Ceccherini-Nelli, and M. Pistello. 2013. Viral vectors: a look back and ahead on gene transfer technology. New Microbiol 36:1-22.

Veron, N., Z. Qu, P. A. Kipen, C. E. Hirst, and C. Marcelle. 2015. CRISPR mediated somatic cell genome engineering in the chicken. Dev Biol 407:68-74.

Voets, O., F. Tielen, E. Elstak, J. Benschop, M. Grimbergen, J. Stallen, R. Janssen, A. van Marle, and C. Essrich. 2017. Highly efficient gene inactivation by adenoviral CRISPR/Cas9 in human primary cells. Plos One 12:e0182974.

Wang, D., H. Mou, S. Li, Y. Li, S. Hough, K. Tran, J. Li, H. Yin, D. G. Anderson, E. J. Sontheimer, Z. Weng, G. Gao, and W. Xue. 2015. Adenovirus-Mediated Somatic Genome Editing of Pten by CRISPR/Cas9 in Mouse Liver in Spite of Cas9-Specific Immune Responses. Hum Gene Ther 26:432-442.

Wang, Y., Y. Ding, and J. Li. 2017. CRISPR-Cas9-Mediated Gene Editing in Mouse Spermatogonial Stem Cells. Methods Mol Biol 1622:293-305.

Watanabe, S., M. Kanatsu-Shinohara, N. Ogonuki, S. Matoba, A. Ogura, and T. Shinohara. 2018. In Vivo Genetic Manipulation of Spermatogonial Stem Cells and Their Microenvironment by Adeno-Associated Viruses. Stem Cell Reports 10:1551-1564.

Wernery, U., C. Liu, V. Baskar, Z. Guerineche, K. A. Khazanehdari, S. Saleem, J. Kinne, R. Wernery, D. K. Griffin, and I. K. Chang. 2010. Primordial germ cell-mediated chimera technology produces viable pure-line Houbara bustard offspring: potential for repopulating an endangered species. Plos One 5:e15824.

Whyte, J., J. D. Glover, M. Woodcock, J. Brzeszczynska, L. Taylor, A. Sherman, P. Kaiser, and M. J. McGrew. 2015. FGF, Insulin, and SMAD Signaling Cooperate for Avian Primordial Germ Cell Self-Renewal. Stem Cell Reports 5:1171-1182.

Wilson, J. M. 1996. Adenoviruses as gene-delivery vehicles. N Engl J Med 334:1185-1187.

Wu, J., and A. Acero-Lopez. 2012. Ovotransferrin: Structure, bioactivities, and preparation. Food Research International 46:480-487.

Yakhkeshi, S., S. Rahimi, M. Sharafi, S. N. Hassani, S. Taleahmad, A. Shahverdi, and H. Baharvand. 2018. In vitro improvement of quail primordial germ cell expansion through activation of TGF-beta signaling pathway. J Cell Biochem 119:4309-4319.

Zabner, J., P. Freimuth, A. Puga, A. Fabrega, and M. J. Welsh. 1997. Lack of high affinity fiber receptor activity explains the resistance of ciliated airway epithelia to adenovirus infection. J Clin Invest 100:1144-1149.

Zeng, W., L. Tang, A. Bondareva, A. Honaramooz, V. Tanco, C. Dores, S. Megee, M. Modelski, J. R. Rodriguez-Sosa, M. Paczkowski, E. Silva, M. Wheeler, R. L. Krisher, and I. Dobrinski. 2013. Viral transduction of male germline stem cells results in transgene transmission after germ cell transplantation in pigs. Biol Reprod 88:27.

Zhang, Y., and J. M. Bergelson. 2005. Adenovirus receptors. J Virol 79:12125-12131.

Zhang, Z., M. B. Renfree, and R. V. Short. 2003. Successful intra- and interspecific male germ cell transplantation in the rat. Biol Reprod 68:961-967.

Zhang, Z., P. Sun, F. Yu, L. Yan, F. Yuan, W. Zhang, T. Wang, Z. Wan, Q. Shao, and Z. Li. 2012. Transgenic quail production by microinjection of lentiviral vector into the early embryo blood vessels. Plos One 7:e50817.

Zhao, C., N. Wu, F. Deng, H. Zhang, N. Wang, W. Zhang, X. Chen, S. Wen, J. Zhang, L. Yin, Z. Liao, Z. Zhang, Q. Zhang, Z. Yan, W. Liu, D. Wu, J. Ye, Y. Deng, G. Zhou, H. H. Luu, R. C. Haydon, W. Si, and T. C. He. 2014. Adenovirus-mediated gene transfer in mesenchymal stem cells can be significantly enhanced by the cationic polymer polybrene. Plos One 9:e92908.

Zheng, Y., Y. Zhang, R. Qu, Y. He, X. Tian, and W. Zeng. 2014. Spermatogonial stem cells from domestic animals: progress and prospects. Reproduction 147:R65-R74.

SUMMARY IN KOREAN

조류는 모델 동물로써 발생학 연구, 질병 저항성 그리고 생체 반응기 모델 등 다양한 응용 가능성을 갖고 있고, 이러한 가능성 때문에 오래전부터 연구되어왔다. 이러한 상황에서 생식선 키메라와 형질전환 조류를 생산하는 것은 매우 가치 있는 연구였고, 조류에서 생식선 키메라와 형질전환을 유도하려는 일련의 연구들이 활발하게 보고되었다. 특히, 닭의 생리적인 특성과 매년 수백 개 이상의 알을 산란한다는 생식 특성 덕분에 조류 형질전환 연구에서 가장 중점적으로 연구되었다. 특히, 조류 형질전환 연구의 중심에서, 닭의 생식선 전이 줄기세포 (germline competent stem cell) 의 한 종류인, 원시생식세포 (primordial germ cell) 에 대한 연구가 활발했으며, 체외에서 장기 배양하는데 성공하였다. 닭을 제외한 다른 조류종에서도 원시생식세포를 분리하고 배양하기 위한 연구가 있었지만, 확보할 수 있는 세포의 수가 매우 적고, 장기간의 체외 배양도 불가능한 수준이었다. 따라서 생식선 키메라 또는 형질전환 조류를 생산하기 위해서 원시생식세포를 대체할 수 있는 다른 종류의 생식선 전이 줄기세포에 대한 연구가 필요하였고, 비교적 포유동물에서 많이 연구된 성체 줄기세포의 한 종류인 정소 줄기세포에 대한 연구가 조류에서도 요구되었다. 본 연구에서는 밀도 구배 원심분리 기법을 이용하여 메추리의 정소세포로부터 정소 줄기세포를 농축시켰고, 농축된 정소 줄기세포를 메추리의 정소에 수술적 기법을 통해 주입함으로써 생식선 키메라를 효과적으로 생산할 수 있었다. 또한, 메추리의 생식선 전이 줄기세포에 아데노바이러스를 사용하여 유전자 표적 편집 기술 (programmable genome editing platform) 의 일종인 CRISPR/Cas9 system 을 도입하였고, 효과적으로 생식선 줄기세포 내에서 효과적으로 유전자 변형을 유도하였다.

첫 번째 연구에서는 메추리 정소줄기세포를 Ficoll-Paque PLUS (Ficoll), Percoll, sucrose 용액에서 밀도 구배 원심분리를 사용하여 분리하였고, qRT-PCR 을 통해서 정소 줄기세포 특이적 유전자 (*GFRA1, ITGA6,* and *ITGB1*) 와 전분화능 관련 유전자 (*NANOG* and *POUV*) 의 발현을 정량 하였다. 흥미롭게도, 세 가지 실험군 모두 상층에 분리된 세포에서 높은 유전자 발현 양상을 보였으며, Ficoll 에 의해 분리된 상층 세포에서 가장 높은 발현을 확인하였다. 이어서 RNA probe hybridzation 과 투과전자현미경을 통해 Ficoll 상층 세포에 정소 줄기세포가 농축되어있음을 확인하였다. 생식선 전이 (germline transmission) 능력을 검증하기 위해 농축된 정소 줄기세포를 buslufan 처리한 메추리의 정소에 수술적 기법으로 주입하였고, 검정 교배를 통해 생식선 전이 효율을 측정하였다. 결과적으로 정소세포를 주입한 대조군 (1.4 ± 1.4 %) 에 비교하여 정소줄기세포를 농축 시킨 후 주입한 실험군 (8.4 ± 1.7 %)에서 약 6 배 높은 생식선 전이 효율을 확인하였다.

두 번째 연구에서는, 분리된 생식선 전이 줄기세포의 유전자 적중 편집을 유도하기 위해 CRISPR/Cas9 system 을 체외에서 도입하였다. 먼저, 아데노바이러스의 외래 유전자 전달 효과를 메추리의 배아 생식선 세포와 농축된 정소줄기세포에서 검증하였다. 또한 세포와 바이러스 사이의 결합력을 증진시키기 위해 중합체의 일종인 poly-L-lysine 을 첨가하여 전달 효과를 최적화시켰다. 이렇게 아데노바이러스를 통해 GFP 유전자가 도입된 원시생식세포를 표면 특이적 마커로 분리하고, 정소 줄기세포와 함께 생식세포 특이적 유전자 (*VASA*, *DAZL*), 전분화능 관련 유전자 (*NANOG*, *POUV*), 정소줄기세포 특이적 유전자 (*GFRA1*, *ITGA6*, *ITGB1*) 의 발현을 검증하였다. 또한 면역 세포 화학 기법을 통해 *DAZL* 유전자를 발현하는 세포에서 아데노바이러스를 통해 들어온 GFP 유전자가 발현되는 것을

확인하였다. 마지막으로, CRISPR/Cas9 system 을 전달하기 위한 아데노바이러스를 제작하였고, 메추리와 생식선 전이 줄기세포에서 *Transferrin* 과 *Hoxb13* 유전자를 적중하여 변형을 유도하였다. T7E1 분석과 염기서열 확인을 통해 적중부위에서 염기서열 변형이 일어난 것을 확인하였고, 메추리의 생식선 전이 줄기세포에서 약 33.3 % 의 효율로 유전자 편집이 일어남을 확인했다. 같은 방법을 닭의 생식선 전이 줄기세포에도 적용하였지만, 아데노바이러스의 전달 효율이 매우 낮았다.

결과적으로, 본 연구에서는 Ficoll 에 의한 밀도 구배 원심분리를 통해서 메추리의 정소 줄기세포를 농축하는데 성공하였으며, 농축된 정소줄기세포의 정소세포로의 이식을 통해 생식선 전이 능력이 향상됨을 검증하였다. 또한 체외에서 아데노바이러스를 통해 CRISPR/Cas9 system 을 메추리 생식선 전이 줄기세포에 효과적으로 전달하였고, 적중하고자한 부위에서 유전자 염기서열의 변형을 확인하였다. 본 연구에서 밝힌 정소줄기세포 농축 기법과 아데노바이러스를 통한 유전자 조절 기법은 여러 멸종위기 조류 종의 복원과 유전자 조절 조류의 생산 연구에 대한 기초 자료로써 기여 할 수 있다.